IDENTIFICATION OF A NOVEL SYNERGISTIC INTERACTION BETWEEN 17 B-ESTRADIOL AND GLUTATHIONE IN NEUROPROTECTION

By

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Evidence proclaiming the beneficial effects of estrogen replacement therapy in neurodegenerative diseases with an oxidative stress component led to the idea that estrogen may be functioning as an antioxidant in neuroprotection. This hypothesis was expanded to include the observation that many antioxidants work in combinations to protect against oxidative stress. Based on these ideas, this dissertation identifies an interaction between 17 β-estradiol (E2) and glutathione (GSH) in neuroprotection.

E2 protection against β-amyloid toxicity (βAP) was tested using SK-N-SH cells, a human neuroblastoma cell line. βAP caused a dose-dependent cell death, with a 20 μM dose killing approximately 50% of the cells at 96 hours of incubation. At 48 and 72 hours, this same amyloid-induced toxicity was correlated with increases in lipid peroxidation. Treatment with E2 at these time points increased viability and was

correlated with marked decreases in lipid peroxidation, indicating an antioxidant mechanism for protection.

Following this observation, the ability of E2 to interact with other antioxidants was established. As expected, supraphysiological concentrations of either E2 or GSH alone were successful in protecting cells from βAP. When a non-protective dose of GSH was added to the cell culture media in the presence of increasing doses of E2, the neuroprotective potency of E2 was shifted to therapeutically relevant doses. Further studies indicate this interaction is specific between E2 and GSH, independent of the toxicity used, and not associated with the tumorigenic nature of the cell line.

Regarding the mechanism of the E2/GSH interaction, the addition of an estrogen receptor antagonist had no effect on neuroprotection; however, the addition of progesterone blocked the response. E2 does not appear to rely on the cytoplasmic concentration of GSH for the protection, as both control cells and cells exposed to βAP showed no difference in GSH content when exposed to E2, GSH, or the combination. Likewise, the enzyme activity was unaffected in this model for both glutathione peroxidase and glutathione reductase. In summary, the mechanism behind this interaction between E2 and GSH remains a mystery; however, its identification should expand the possibilities for potential treatment strategies for neurodegenerative conditions.

CHAPTER 1
RATIONALE

Women entering menopause are at an increased risk for osteoporosis [43], cardiovascular disease including stroke [74], and Alzheimer's disease (AD)[158]. The economic burden associated with these "postmenopausal diseases" is high. Recent articles estimate direct costs associated with hip fractures at $11.6 billion [102], stroke at $17 billion [208], and AD at $20.6 billion [56]. Because the time period of increased risk correlates with an estrogen-deprived state, the possibility that a lack of estrogen contributes to the pathogenesis of these diseases has been acknowledged and, conversely, that estrogen replacement therapy (ERT) may indeed be worthwhile in treatment or disease prevention.

Beneficial effects of estrogens in the reduction of stroke mortality have been examined in epidemiological studies. One such study, where the main outcome measure was the incidence of stroke either fatal or non-fatal, demonstrated a decrease in the risk of stroke incidence and mortality in white postmenopausal women on ERT [61]. A thorough review of stroke and the effects of ERT found 19 studies with relative risk ratios from 0 to 2.3, yet it was concluded that the bulk of evidence suggested ERT decreased the risk of stroke [156]. In a study looking at both the incidence of new cardiovascular events (e.g., new hypertension, ischemic changes on an electrocardiogram, an occurrence of myocardial infarction, or cerebrovascular accidents) and osteoporosis (including loss of height, mean

cortical bone density, vertebral compression, and peripheral fractures) women on ERT had a significantly lowered risk with regard to cerebrovascular accidents, peripheral fractures, and vertebral compression [114]. Furthermore, early studies on osteoporosis demonstrated that reduction in cortical bone loss by ERT depended on the dose administered [93] and that long term estrogen use was correlated with a 53% lower incidence of osteoporotic fractures [58].

Epidemiological evidence for ERT in the prevention of AD is mounting (for review see [159]). Four studies have provided prospective data before the onset of dementia symptoms [26,104,157,206] and three of these studies found reduced risk for AD (35%-60%) with ERT [104,157,206]. Like the osteoporosis studies, a dose response effect was found in the Leisure World cohort such that women taking low dose estrogen ($\leq$0.625 mg) were at a higher risk (0.78) of developing AD while high dose estrogen ($\geq$1.25 mg) had a lower risk (0.54) [157].

Looking at disease treatment instead of disease prevention, several studies have examined the effects of estrogens on AD. Regardless of small numbers of subjects, short duration of treatments, and fairly brief cognitive screening tests, positive outcomes were demonstrated [59,60,92,150]. Only one study failed to show cognitive improvement [216]. Estrogens have not been approved in the treatment of stroke, but animal models of stroke show beneficial effects of estrogens when administered up to 40 minutes after stroke induction [224].

In closing, stroke is the third leading cause of death in the US, while AD is the fourteenth [3] . AD has an increasing incidence after age 65 [7] and afflicts women 2-3

times more than men [5]. The burden of AD falls particularly hard on women because women live an average of 6 years longer than men [3]. The Food and Drug Administration has approved only two drugs for AD treatment, tacrine (Cognex®) and donepezil (Aricept®) [47]. These are centrally active cholinesterase inhibitors and while they may improve cognitive symptoms, benefits are modest and accompanied by undesirable side effects. Further, beneficial effects in the case of Cognex® were increased when given with estrogens [180]. There is a great need for treatment as well as primary prevention of these neurodegenerative diseases and given the dose and time dependence relationships, the evidence for beneficial effects with ERT is substantial. Understanding the mechanisms behind these beneficial effects may provide insight into developing new drugs which lack side effects, and ultimately decrease the number of patient populations (such as men or women at risk for breast cancer) where estrogen replacement is contraindicated.

CHAPTER 2
LITERATURE REVIEW

Therapeutically, estrogens have been used for contraception in cycling women and for hormone replacement in surgical menopause and post-menopausal women, although the forms and doses of estrogens differ. The use of estrogens as pharmacological agents in these circumstances has directly resulted from the understanding of their physiological actions. The future use of estrogens in treating neurodegenerative diseases depends on our understanding of their actions in the brain. When women are most susceptible to cerebrovascular accidents and AD, i.e., neurodegenerative conditions which are correlated with an increase in oxidative stress, they are hypoestrogenic. The protection afforded by estrogen replacement therapy (ERT) may reside in the antioxidant potential of estrogens, given their phenolic nature. This dissertation is based on the hypothesis that estradiol, at physiologically relevant doses, protects against insults which may occur in neurodegeneration by cycling, or perhaps recycling, with other molecules specifically glutathione. The following chapter is devoted to discussing: (1) the physiological actions of the ovarian hormones to include estradiol; (2) the functions of antioxidants such as glutathione; (3) the integration of oxidative stress as a component of neurodegenerative disease; and (4) the protection afforded by estrogens in a variety of animal and cell culture models that support the therapeutic effects of ERT previously discussed.

2.1 Steroidogenesis and Physiological Action

The 1920's brought the first reports of a female sex hormone in the blood of various animals [118] and the identification of the hormonal function of the corpus luteum [39]. These early investigations revealed that the ovary secretes both estrogens and progestins in premenopausal women. Estradiol levels in cycling women are between 50-200 pg/ml, while in post menopausal women these levels plummet to 3-6 pg/ml.

Functional ovaries rely on the initiation of meiosis, follicle formation, and steroidogenic cell differentiation early in fetal development [29]. At this time, the ovaries are covered by germinal epithelium and as the fetus develops, primordial ova differentiate from the germinal epithelium and migrate to the ovarian cortex. Each ovum then collects around it a layer of cells from the ovarian stroma, which subsequently take on epithelioid character and become what are known as granulosa cells. When the ovum is surrounded by a layer of granulosa cells, it is called a primordial follicle [29]. At the 30^{th} week of gestation, the number of follicles reaches about 6×10^6, but will degenerate to only about 1×10^6 at birth for each ovary [9]. This number will further decline to $0.3\text{-}0.4 \times 10^6$ available ova at puberty.

Puberty marks the beginning of reproductive cycling, although factors controlling the onset remain an enigma. Throughout reproductive cycling, approximately 400 of these follicles will develop a second class of cells, called the theca, in response to hormonal stimulation received from the pituitary. The theca is divided into two sublayers, the theca interna which secretes androgenic precursors for estrogen synthesis performed by the granulosa cells, and the theca externa, which becomes the capsule of the developing

follicle. Several follicles may enlarge but for unknown reasons only one follicle is recruited each month to undergo higher development while the remainder undergo atresia. In response to a series of hormonal events, the recruited follicle expels its ova during ovulation, and subsequent luteinization of the granulosa cells develops into the corpus luteum. If fertilization occurs, then the production of chorionic gonadotropin maintains the luteal environment for early pregnancy. Without fertilization, hormone production declines and the corpus luteum involutes, thus losing its secretory function and becoming corpus albicans. Menstruation begins at this point, setting the stage for a new ovarian cycle. The menstrual cycle is divided into two phases: the follicular phase, which is highlighted by the recruitment of the dominant follicle, and the luteal phase, which occurs after ovulation. Estrogens are secreted by granulosa cells into follicular fluid, and levels are highest during the late follicular phase [8]. Estradiol synthesis by granulosa cells requires aromatase enzyme activity, which is induced by the gonadotropins (FSH and LH) released from the anterior pituitary, and androgenic substrates derived from theca cells. Over 90% of the circulating estrogens secreted during late follicular phase originates from the dominant follicle [8] .

The aforementioned androgenic precursors can be either androstenedione or testosterone. An aromatase catalyzes the aromatization of the A ring in three steps using 3 molecules each of NADPH and molecular oxygen as cosubstrates [137]. The aromatase activity resides within a transmembrane glycoprotein, and NADPH-cytochrome P450 reductase is also essential. Co-localization of both proteins can be found in ovarian granulosa cells, testicular sertoli and leydig cells, adipose stromal cells, and various brain regions.

In the luteal phase, progesterone is the major secretory product although estrogens remain elevated. Progesterone limits the proliferative effect of estrogens on the endometrium by stimulating differentiation and is important for implantation. Another prime target for progesterone is the mucosal lining of the genital tract. For progesterone to affect the genital tract, the cells must first be exposed to estrogens which induce the formation of progesterone receptors. If pregnancy does not occur, the corpus luteum regresses, estrogen and progesterone levels fall, and menstrual discharge results because the endometrium cannot be maintained.

2.2 The HPG Axis

The menstrual cycle is under neuroendocrine control, involving the hypothalamic-pituitary-gonadotropin axis. The pulse generator in the arcuate nucleus of the hypothalamus periodically releases gonadotropin-releasing hormone (GnRH) into the portal vasculature of the pituitary gland. GnRH then acts on the gonadotropes of the pituitary to cause the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. The gonadotropins (LH and FSH) are responsible for the growth and maturation of the Graafian follicle in the ovary, for the production of estrogen by the granulosa cells, and production of progesterone by the corpus luteum once a mature follicle has been recruited. Progesterone exerts feedback regulation on the pituitary and hypothalamus, and estrogen exerts feedback regulation on the pituitary [116].

Evidence that neuronal structure is altered by estrogens has been demonstrated in the hypothalamus [32] and the hippocampus [73,219]. It is interesting that the

hippocampal formation is particularly sensitive to gonadal steroids as it plays a major role in memory and cognition. Work by McEwen and colleagues shows that administration of estrogens can increase dendritic spine density when compared to ovariectomized animals, while progesterone causes a decrease in spine density [219]. Extrapolated to behavior, changes in memory function have been demonstrated across the menstrual cycle [187] and after surgical menopause [186], raising the possibility that there is a role for estrogen in maintenance of neuronal function.

2.3 Reproductive Senescence

It is clear that the roles of the brain and the ovary in cycling women are highly intertwined, yet their relative contributions to reproductive senescence are not well understood. One theory, offered by Richardson and Nelson [170], draws on the idea that menopause is driven by follicular depletion. This is supported by work showing an accelerated depletion of follicles so that within one year of menopausal transition there are few to none remaining [170]. If this theory is correct, then the events in the ovary drive the menopausal transition. Another theory by Wise et al. [218] suggests that the menopause is driven by the brain, and changes in rhythmicity and cycling are driven by changes in the function of the hypothalamus. Santoro [179] attempted to sort out whether menopause itself or aging has the predominant effect on endocrine function by comparing women with age appropriate menopause to women with premature ovarian failure. Conclusions from this study support the idea that the deficits in hypothalamic GnRH secretion, pituitary sensitivity or gonadotropin reserve are the underlying cause; however, it cannot be ignored that ovarian status may prejudice that conclusion.

Regardless of the reason for the menopausal transition, estrogen levels in post menopausal women are low, decreases in LH are noted, and a monotropic rise in FSH is well established. Ovarian failure restricts estrogen production to adipose stroma. If ERT is obtained, the form of estrogen, the route of administration, and the addition of progesterone differs among women. Equine estrogens are most frequently prescribed and contain several estrogenic molecules; thus the beneficial effects resulting from ERT may be driven by a variety of molecular mechanisms.

2.4 Molecular Effects of Steroid Hormones

The molecular actions of steroids may rely on the presence of a nuclear or membrane receptor. Studying the genomic actions of steroids began when it was acknowledged that estrogens could stimulate nucleotide synthesis and control gene expression [142]. The first reference to the possibility of an estrogen receptor molecule was by Jensen and Jacobson when they showed that estrogens are more readily accumulated and retained in estrogen responsive tissues than in non-responsive tissues [100]. When the location of estrogen binding was examined, 56% of estrogens were found in the nuclear-myofibrillary fraction [148]. Notebook and Gorski further demonstrated competitive inhibition for estrogens, and estrogen release from the nuclear fraction was dependent on proteases and extreme pH and not nucleases, suggesting that binding in the nucleus was to a protein specific for estrogen molecules. Unoccupied estrogen receptor was localized to the nucleus [108,129,217]. Thus, the classical action of estrogens depends on a intra nuclear receptor and the binding of the steroid to it, and has been the subject of extensive reviews [84,153].

The estrogen receptor (ER) is complexed with heat shock and other proteins in its inactive state ([33,182]for review see [197]) which are speculated to aid the protein in proper folding. It will shed these proteins when exposed to its ligand, resulting in a conformational change which reveals the DNA binding domain [12]. The phenolic nature of the A ring is the principal structural feature responsible for the affinity of ligand for the ER. The steroid-receptor complex dimerizes with another steroid-receptor complex, and as a homodimer binds to the estrogen response element on the DNA and initiates gene transcription [111].

Conversely, the existence of a membrane-bound steroid receptor for estrogen was first reported in uterine and liver tissue by Pietras and Szego [164] and in synaptic plasma membrane by Towle and Sze [211]. Justification for the non-classical actions of estrogens came from reports of rapid effects (from seconds to minutes) [50], and conjugation to large proteins such as bovine serum albumin (BSA) inhibiting cell entry still elicited a response (for review see [25]). Further, protein synthesis inhibitors could not block the hyper-polarization resulting from estrogen exposure [146]. Co-localization of estrogen binding with p75, a low affinity neurotrophin receptor suggested that estrogens may influence cells at the level of signal transduction or gene transcription via crosstalk with a growth factor associated pathway [139,210]. This is also supported by the co-expression of ER mRNA with mRNA for neurotrophins and their receptors [138], along with the demonstration that estrogen regulates their expression [201]. It has been shown in MCF-7 breast cancer cells that tyrosine kinase cascades involving $p21^{ras}$ are activated by estrogen receptor complexes [135]. In SK-N-SH neuroblastoma cells, rapid membrane effects of

estrogens conjugated to BSA on the Ras/Raf/MEK/ERK pathway have been recently demonstrated [215].

It was once thought that the ER gene deletion would be lethal; however, estrogen receptor knock out mice were engineered and with the exception of infertility problems were otherwise healthy [120]. The subsequent cloning of a novel estrogen receptor, ER β, from rat prostate was identified in the rat ovary [110], and a variety of brain regions [189]. Most recently, cell membrane and nuclear estrogen receptors have been shown to be derived from single transcripts and this applies to both ER α and ER β [168]. Interestingly, the membrane estrogen receptors, both α and β, activate G-proteins, ERK, and cell proliferation; however, there is a differential regulation of C-jun kinase [168].

Allusion to C-jun kinase brings forth yet another possible mechanism involving the ER-ligand bound complex: activation of the transcription of a number of early response genes. The binding of one ER-ligand complex with fos or jun can activate the AP-1 response element [67,212]. This promiscuous binding of the ER complex with other proteins for gene transcription reveals additional levels of complexity in estrogen mediated cell signaling. Included in this complexity is the differential ligand activation of ERα and ERβ at AP-1 sites [155]. These discoveries warrant the re-examination of the involvement of the estrogen receptor in non-classically mediated events.

The stability of transcription in response to estrogens or the effects of signal strength and duration of exposure have not been addressed. The importance of these factors is exemplified by studies using PC 12 cells which can either differentiate or proliferate in response to growth factor stimulation according to time or strength of

stimulus (for review see [124]). Since these pathways are involved in estrogen signaling, these factors should be evaluated. Jensen and Jacobsen [100] may indeed be right on the mark:

> To a tissue that's trying to grow
> We hope these experiments show
> With steroids phenolic,
> Don't get metabolic,
> Just grab on, and never let go.

2.5 Oxidative Stress as a Biological Process

Aerobic metabolism results in oxidative stress, the products of which can cause damage to cellular elements including nucleic acids, proteins and lipids. Ground state oxygen, by virtue of having two unpaired electrons in its outer orbital, can generate molecules that contain one or more unpaired electrons in their outer shells, i.e. free radicals. The reduction products at the one-, two-, and three- electron steps are superoxide radical, hydrogen peroxide, and hydroxyl radical, respectively, and all are undoubtedly produced in biological systems [105].

$$\mathbf{O_2} \Rightarrow \mathbf{\cdot O_2^-} \Rightarrow \mathbf{H_2O_2} \Rightarrow \mathbf{\cdot OH} \Rightarrow \mathbf{H_2O + O_2}$$

step 1 step 2 step 3

More recently, the collective term reactive oxygen species (ROS) has been used, and designates molecules with chemically reactive oxygen groups involved in free radical chemistry, both oxidation and reduction reactions. These molecules are highly reactive,

have short half lives, and are implicated in cellular injury and tissue damage [87]. Paramount to the production of ROS are the transition metals, namely iron (Fe), copper (Cu), and manganese (Mn), that participate in oxidation and reduction reactions by virtue of their ability to change valence in their outer d orbitals. While this ability makes these ions important as cofactors necessary for cellular function, it simultaneously renders them detrimental if not highly bound. Because hydrogen peroxide, in addition to being freely permeable to the cell membrane [87], is a by-product of aerobic metabolism and both Fe and Cu are found in the central nervous system, it is plausible that the hydroxyl radical can be formed *in vivo* providing that both entities are in the same cellular compartment. A mélange of radical forming reactions exists (e.g., the Fenton, Haber-Weiss, and Maillard reactions), the aftermath being lipid peroxidation, protein carbonyl formation and DNA strand breakage.

The presence of high concentrations of unsaturated fatty acids along with their post-mitotic state and increased oxidative metabolism makes neurons highly susceptible to attack by ROS. Lipid peroxidation can be described as the abstraction of a hydrogen ion from a polyunsaturated fatty acid, producing a lipid radical and resulting in a cascade of events, which can be self propagating, generating organic peroxides, conjugated dienes, aldehydes, and ketones (e.g. malondialdehyde and 4-hydroxynonenal). Peroxidation of unsaturated fatty acids makes them more hydrophilic, thus altering the membrane structure and affecting transporters and membrane receptors. While it is debatable whether lipid peroxidation is a primary event or one that is more downstream in the free radical cascade, it is agreed upon that compromising the integrity of the membrane has deleterious consequences [87].

Protein carbonyl groups (the introduction of aldehyde and ketone groups to proteins by a variety of oxidative reactions) are found on proteins subject to oxidative damage. This occurs by the combined effects of H_2O_2 and trace metal cations, namely Fe and Cu, which can produce the hydroxyl radical, or secondarily by the oxidation of other molecules which in turn oxidize proteins. The amino acids lysine, proline, histidine and arginine have been found to be the most sensitive to oxidative damage, and -SH groups found on proteins are also susceptible. The relevance of these reactions has been demonstrated as excess brain protein oxidation was found in normal aging and in Alzheimer's disease [31].

The molecular mechanisms responsible for DNA damage include the indirect activation of nucleases due to oxidative stress and the direct reaction of hydroxyl radicals with the DNA [86]. It is thought that the metal ions present in DNA react with freely permeable H_2O_2, causing hydroxyl ion production, with subsequent strand breakage, either single or double stranded, base hydroxylation, and/or chromosomal aberrations.

2.6 Defenses to Oxidative Stress

The elimination of free radicals occurs when two radical species react together, resulting in a termination reaction, or when the cells's defenses detoxify the radicals produced as a result of aerobic metabolism or assault by another molecule. Enzyme systems which operate to detoxify ROS include the superoxide dismutase family (SOD), catalase (CAT) and glutathione peroxidase (GPX).

$$\cdot O_2^- \xrightarrow[\text{SOD}]{} H_2O_2 \xrightarrow[\text{CAT|GPX}]{} H_2O$$

The superoxide dismutase family is composed of the constitutively expressed CuZnSOD, first identified by McCord and Fridovich [130], the inducible MnSOD, and an extracellular SOD which in mammals is a tetrameric form of CuZnSOD instead of a dimer. SOD is important in free radical defense because it catalyzes the conversion of superoxide radicals into hydrogen peroxide.

Catalase (CAT) acts on the production of hydrogen peroxide to allow the formation of water. This reaction is important because hydrogen peroxide allowed to roam unchecked will result in oxidizing reactions. The seriousness is compounded by the ability of H_2O_2 to cross membranes. Glutathione peroxidase can also catalyze this reaction and in addition can repair membrane lipids in the event of lipid peroxidation. This reaction involves the use of glutathione (GSH) as the donor for repair, and the regeneration of GSH comes from glutathione reductase and NADPH produced from the hexose monophosphate shunt.

Naturally occurring antioxidants, such as taurine, lipoic acid, ascorbic acid, α-tocopherol, GSH, melatonin, and estrogen, working alone or in concert with these systems, further enhance the body's defenses. It is likely that an antioxidant is multifaceted in the cell's protection. For example, the glutathione (GSH) system operates in several ways for cellular defense [133]. First, it participates in the non-enzymatic reduction of free radicals. Second, it conjugates directly with toxins. Third, it acts on intracellular peroxides via GSH peroxidase and GSH S-transferases. Finally, it maintains disulfide bonds in proteins in their reduced forms, utilizing GSH S-transferases.

Regeneration or recycling of any of these systems would be beneficial as the brain has little to no catalase to speak of, and only moderate amounts of glutathione peroxidase to contend with hydrogen peroxide production [87]. Recycling schemes have been demonstrated for α-tocopherol and ascorbic acid, α-tocopherol and ubiquinol, and ascorbic acid and glutathione.

2.7 Neurodegenerative diseases and Oxidation

Ischemia/reperfusion injury which occurs in stroke is made up of two very different components. An ischemic event occurs when tissues are deprived of oxygen, with the amount of damage depending on the duration, the tissue, and the extent of oxygen deprivation. Reperfusion injury is the damage that occurs to an organ during the resumption of blood flow. Both ischemia and reperfusion lead to oxidative stress: The mitochondria become leaky as oxygen is not available to act as the terminal electron donor in the respiratory transport chain releasing free radicals into the cytoplasm, and ATP is not generated in sufficient quantity to maintain membrane potentials. Transport of substrates for antioxidant molecules are decreased, and AMP accumulates and is metabolized to hypoxanthine, a substrate for xanthine oxidase. Xanthine oxidase requires oxygen, and subsequently when reperfusion takes place and oxygen becomes available for use with this enzyme, an elevated number of free radicals are produced. Since both phases are associated with free radical accumulation, antioxidants and antioxidants defenses may be important in assuaging this condition.

With regard to Alzheimer's disease (AD), the diagnosis of AD relies on the demonstration of senile plaques and neurofibrillary tangles found in the post-mortem

examination of brain tissue. At the cellular level, both neurofibrillary tangles and senile plaques stain with antibodies to superoxide dismutase and catalase [65], indicating these neurons may have been experiencing an oxidative stress. In addition, neurons bearing neurofibrillary tangles stain with an antibody to protein carbonyls [198] and demonstrate the presence of nitrotyrosine, another signal of oxidative stress [70]. Further evidence demonstrates major oxidative damage in the brains of AD patients. Lipid peroxidation, as assessed by malondialdehyde concentration, was shown to be increased in multiple studies [10,160], with spin trapping techniques demonstrating oxidative damage to both lipids and proteins [31] and HPLC techniques showing increased oxidative damage to both nuclear and mitochondrial DNA [131].

A constitutive protein found in senile plaques is a 40-43 amino acid peptide called β-amyloid, identified by Glenner and Wong in 1984[68]. *In vitro* results showing aggregated ß-amyloid to be cytotoxic and oxidative [165,223] add support to the aforementioned human studies. A ten amino acid portion of this protein, βAP 25-35 (βAP) has been demonstrated to be the toxic portion of the molecule [222], requiring contact with the plasma membrane [127] and has the added feature of rapid aggregation.

In all probability, the increased oxidative damage results in either increases in free radical production or decreases in the ability to defend against free radicals or both. Sources of increased free radicals would result from: 1) a deficiency of cytochrome oxidase IV [11,145,161,162]; 2) a direct effect of βAP [28,90] , the demonstrated breakdown of the mitochondria as a result of βAP treatment [13], and the generation of H_2O_2 resulting from a βAP challenge [14]. Since increased antioxidant enzyme activity

accounts for the survival of βAP resistant cells [177], the importance of an intact defense is emphasized.

2.8 Estrogen and Neuroprotection

In vivo studies show that estrogens can protect basal forebrain cholinergic neurons from the degenerative effects of fimbrial lesions [166] and also reduce the size of infarct by 50% [188,192,224]. Additionally, *in vitro* studies utilizing cell lines and rat cortical neurons demonstrate protective actions of estrogens using serum deprivation [75], exposure to βAP [16,71] and glutamate toxicity [16,71,194]. Structure activity relationships demonstrate that an intact phenolic A ring and at least three rings of the steroid structure are necessary for neuroprotection [15,76]. Given the phenolic nature of a variety of antioxidants, the structural requirements for neuroprotection indicate that this may be one component of estrogen's neuroprotective actions.

In tests of a variety of oxidizing systems, Lacort et al.[113] demonstrated that supraphysiological doses of estrogens can interact with the peroxidative process at different levels, and offer that the interactions of estrogens with iron or metal-derived species are the most important mode of inhibition. Ruiz-Larrea et al. [175] directly tested the effects of estrogens on the redox chemistry of iron in a cell free system and determined that the inhibition of peroxidation by estrogens was directly related to their ability to decrease the oxidation of Fe (II) to Fe (III) in aqueous solutions. Mooradian [141] shows that, in a cell free system, 17 β-estradiol (βE2) 17α-estradiol (αE2) and estriol can delay the quenching of fluorescence exhibited by the peroxy radical, indicating a scavenging effect; however, other steroids tested lacking a phenolic A ring were either

mildly pro-oxidant or had no effect. Together, the data suggest a correlation between antioxidant and neuroprotective properties.

Estrogen administration to post-menopausal women is correlated with a decrease in low density lipoprotein (LDL) oxidation [176]. In rabbits, estrogenic molecules which lack activity at the estrogen receptor also decrease LDL oxidation [136]. Estrogens were shown to be more potent antioxidants than α-tocopherol when lipid peroxidation was measured in liver microsomes [136,143,147]. Sugioka et al.[203] postulate that this relates to its phenolic A ring showing that estrogens can also inhibit lipid peroxidation in rat liver microsomes and Miller [136] offers that by flanking the 2 or 4 position with large alkyl groups its possible to increase the antioxidant potential *in vivo.*

Jellnick and Bradlow [98] used tritium incorporated into the steroid molecule to demonstrate that the donated hydrogen ion which inhibits the oxidative cascades can originate from several positions on the A ring of estrogens. This may in part explain the stoichiometry of the loss of neuroprotective activity with the methylation of the phenolic A hydroxyl group of estrogens [76]: steric hindrance of the other A ring positions may prevent their donation of hydrogen ions.

In monocytes, βE2 was found to be the most potent estrogen in decreasing cell mediated and copper-induced modification of LDL [128]. Rifici evaluated the effect of βE2 on superoxide production in monocytes and found no effect [172]. Lastly, it is likely that estrogen and its metabolites may have different affinities for the distinct lipid substrates in the inhibition of lipid peroxides [205]. This observation may provide an explanation for the difference in the potency of estrogens in the various models for antioxidant and protective activities.

There is a correlation between estrogen's ability to decrease oxidation and increase cell survivability [16,71]. *In vitro* cell culture models showing protective effects of estrogens to oxidative insults utilizing βAP induced toxicity demonstrate concomitant decreases in lipid peroxidation, at μM doses [16,71].

Entertaining the notion that antioxidants work in concert with each other brought forward the working hypothesis that estrogen is acting in a cycling fashion with another antioxidant, thereby decreasing free radicals which may accumulate by a variety of mechanisms. Depletion of estrogen as happens in the menopause would contribute to oxidative stress due to the decline in antioxidant defense. Glutathione is a likely candidate to interact with estrogen given its ability to repair the plasma membrane and the lipophilic character of estrogens would partition them to this cell structure. This dissertation is designed to elucidate the possible mechanisms behind this interaction.

CHAPTER 3
CELL CULTURE TECHNIQUES AND ASSAYS

The vast majority of the work in this dissertation relies on cell culture methods using SK-N-SH neuroblastoma cells and primary rat cortical neurons. This chapter will deal with the materials, techniques, and the assays employed in these studies.

3.1 Primary Rat Cortical Cultures

Primary neuronal cultures were prepared according to methods described elsewhere [34]. Briefly, Female Sprague-Dawley rats were housed and bred in our animal facility. Primary cortical neurons were prepared from 1-day-old rat pups as follows: brain tissue was removed from rat pups and placed in isotonic salt solution containing 100 U of penicillin G, 100 µg of streptomycin, and 0.25 µg of amphotericin B (Fungizone, Life Technologies) per ml (pH 7.4). After removal of blood vessels and pia mater, the tissue was sectioned into approximately 2-mm chunks, suspended in 25 ml of 0.25% trypsin (weight/volume) in isotonic salt solution (pH 7.4), and placed in a shaking water bath for 10 min at 37° C to dissociate the cells. The dissociated cell suspension was then removed and combined with 10 ml of DMEM containing 10% plasma-derived horse serum (PDHS, Central Biomedia, Irwin, MO) and the undissociated chunks were mixed with 160 µg of DNAse 1 and triturated until the cells dissociated. The cell suspensions were then combined, centrifuged at 1000 x *g* for 10 min, and the resulting cell pellet washed with 50 ml DMEM with 10% PDHS and plated on poly-l-lysine pre-coated 35 mm culture dishes at a density of 4×10^6 cells per dish and incubated in a humidified incubator containing

95% air and 5% CO_2 at 37° C. On day 3, cells were treated with β-cytosine arabinoside (10 μM) for 48 h and media was then aspirated and replaced with DMEM containing 10% PDHS and incubated for an additional 5 days before being used in experiments. At this time, cultures contained approximately 90% neurons and 10% astroglia. These appeared as many phase contrast bright cells with characteristic neuronal morphology overlaying a number of flat phase dark cells which had typical astroglial morphology.

Viability determinations were made on primary cultures after 24 h of incubation with various treatments using the LIVE/DEAD® viability/cytotoxicity kit (Molecular Probes, Eugene, OR) according to manufacturer's instructions. Basically, the calcein AM (5 μM) and ethidium homodimer (5 μM) dyes were made fresh prior to use, and 300 μl were used to cover the bottom of the culture dish. Live cells were distinguished by the presence of intracellular esterase activity, which cleaves the calcein AM dye, producing a bright green fluorescence when excited. Ethidium homodimer enters cells with damaged membranes, and upon binding to nucleic acids, produces a red fluorescence. Both dyes are excited at 485 nm, and cultures plates were viewed with a fluorescent microscope (Nikon Diaphot-300). Three fields were photographed on high dry magnification after randomly moving the dish and focusing, and the average number of live cells per field was determined by counting the number of bright green cells.

3.2 SK-N-SH Neuroblastoma Cell Cultures

SK-N-SH neuroblastoma cells were obtained from American Type Tissue Collection (Rockville, MD) in passes 24-31 and used in passages 28-60 for the experiments herein. Cell cultures were grown to confluency in RPMI-1640 media

supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin (all reagents obtained from Sigma Chemical Corporation, St. Louis, MO) in monolayers in plastic Corning 150 cm^2 flasks (Fisher Scientific, Inc. Orlando, FL) at 37° C under 5 % CO_2 and 95 % air. Media was changed three times weekly. Cells were observed with a phase contrast microscope (Nikon Diaphot-300). Experiments were initiated at back culture and plated at 1 x 10^6 cells/ml for viability assays.

Cell viability was determined utilizing the trypan blue exclusion method [21]. At the appropriate times, treatment media was decanted and cells were lifted by incubating with 0.4 ml 0.02% EDTA (Sigma) or versene (Life Technologies, NY) for 30 min at 37° C. Cells were suspended by repeated pipetting. One-hundred μl aliquots from each cell suspension were incubated with 100 μl aliquots of 0.4% trypan blue stain (Sigma Chemical Corp.) for 5 min at room temperature. All suspensions were counted on a Neubauer hemacytometer within 10 minutes of trypan blue addition. Two independent counts of live and dead cells were made for each aliquot.

3.3 Glucose/Lactate Analysis

Glucose and lactate determinations were assayed using a YSI STAT 3000 glucose analyzer (YSI Inc., Yellow Springs, OH) on initial and decanted treatment media. Utilization was determined per million trypan blue excluding cells.

3.4 Thiobarbituric Acid Reactive Products (TBARs)

At the appropriate times, cell suspensions, as generated for cell viability, were tested for the production of malondialdehyde (MDA), a by-product of lipid peroxidation which can be assayed using a modified TBAR procedure based on methods used by

Subbarao et al. [202] and Ohkawa et al. [149]. Two-hundred µl of cell suspension was added to 300 µl of 0.9% normal saline in glass tubes, and incubated with 0.5 ml 20 % glacial acetic acid and 1.0 ml 0.8% thiobarbituric acid for 60 minutes (lightly stoppered) in a boiling water bath. Upon cooling, tubes were centrifuged for 10 minutes at 1500 rpm and then 200 µl pipetted into 96 well plates and read on the SLT 3000 tray reader at 530 nm. Thiobarbituric acid (Sigma Chemical Corp.) was initially made at 0.8%, and used in the assay to give a final concentration of 0.4%. Acetic acid (20% v/v; Fisher Scientific) was adjusted to a pH of 3.5 with the addition of NaOH and used at a final concentration of 5%. External standards used in the thiobarbituric acid reactive product procedure (TBARs) were made from 1,1,3,3-tetramethoxypropane (TMP, Sigma Chemical Corp.) in reagent grade ethanol (Fisher Scientific) and diluted in 0.9% normal saline for generation of standard curves.

3.5 Glutathione Content Assay

Glutathione content was analyzed using the Tietze method [209] with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB or Ellman's reagent). George Ellman first used this reagent to determine tissue sulfhydryl content when it was noticed that some drugs might affect cellular thiol status [54]. Tietze based his assay for glutathione on DTNB for detection, and exploited the ability of glutathione to be enzymatically reduced by the enzyme glutathione reductase discovered by Rall and Lehninger in 1951 [167]. The Tietze assay detects total glutathione content, which includes reduced glutathione (GSH), glutathione disulfide (GSSG) and possibly glutathione in disulfide linkages with other soluble thiols. Glutathione becomes sequentially oxidized by 5,5'-dithiobis (2-nitrobenzoic

acid) (DTNB) and reduced by NADPH in the presence of glutathione reductase (Fig. 3.1). The rate of 2-nitro5-thiobenzoic acid formation is monitored for 6 minutes at 412 nm and can be followed visually by an increasingly yellow color. The amount of glutathione present in the sample is evaluated using a standard curve. GSSG content can be obtained if the reduced GSH is derivatized from the sample. Analysis of GSSG was based on modifications of the Tietze method by Griffith [81] who used 2-vinylpyridine for derivatization.

For cell culture, SK-N-SH neuroblastoma cells were plated at 4 x 10^6 cells/plate and incubated in 100 mm dishes until 80% confluency was obtained. At this time, media was decanted and treatments added as appropriate. Following treatment for 24 h, cells were incubated in 0.9 ml versene for lifting, triturated to ensure even pipetting, and subsequently divided into three equal aliquots, one each for cell viability, total glutathione and GSSG assay.

For the total glutathione and GSSG assay, cells were spun at 1500 rpm for 10 minutes and the supernatant removed. Cells were then resuspended in 250 µl of 1% sulfasalicylic acid to precipitate proteins, sonicated to break open cell membranes, and centrifuged at 3000 rpm for 15 minutes at 4° C. The supernatant was again removed and assayed for total glutathione. Determination of GSSG required aliquots of the supernatant be transferred to a clean microcentrifuge tube and derivatized with 2-vinyl pyridine for 60 min at a concentration of 18 mM (2 µl of 97% 2-vinylpyridine for every 100 µl of sample). The pH was checked for each sample as the reaction proceeds optimally at pH 6-7. If the pH was too low, then 2-vinylpyridine was added increments of 2 µl until the obtained pH

was in the desired range. Samples were run using the following recipe: 0.6 ml buffer, up to 0.2 ml sample in SSA depending on GSH concentration, 0.1 ml DTNB and 0.1 ml NADPH/ Glutathione Reductase enzyme mixture.

Standard curves are generated using 0-250 ng/ml of GSH or 0-50 ng/ml of GSSG of standards made in buffer and derivatized accordingly. Two-hundred µl of sulfasalicylic acid were added to all standards and buffer amounts were adjusted to accommodate standard volumes. Glutathione concentrations were normalized for total number of cells in the sample to allow for comparison between groups.

3.6 Cell Extraction Protocol for Enzyme Activity Assays

The cell extraction protocol was based on methods described by Schreiber et al. [181]. Cells collected from 100 mm plates by lifting in 0.9 ml versene (Life Technologies) were pelleted using a microcentrifuge. The versene supernatant was removed and the cells resuspended in 600 µl buffer containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT with 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 5 µM microcystin. The cells were allowed to swell on ice for 15 minutes after which 25 µl of the cell lysing agent NP-40 was added and vortexed vigorously for 10 seconds. The supernatant was divided among two microcentrifuge tubes, one each for glutathione peroxidase and glutathione reductase activity assays. The nuclear pellet was stored in high salt buffer containing 0.05 M $NaPO_4$, 2.0 M NaCl, 0.002 M EDTA, pH 7.4 and frozen at -80° C until DNA content determined.

3.7 Cellular Glutathione Peroxidase Activity

Cellular glutathione peroxidase (GPX) activity was obtained using a kit supplied by Calbiochem (San Diego, CA). Basically, GPX activity is measured indirectly based on the oxidation of NADPH to $NADP^+$. The reaction is initiated after the introduction of an organic hydroperoxide, which, utilizing the GPX and reduced GSH as a substrate, produces GSSG. The GSSG is reduced back to GSH using the enzyme glutathione reductase with NADPH as a substrate. The oxidation of NADPH to $NADP^+$ is monitored for three minutes via the absorbance at 340 nm, and the rate of decrease measured corresponds to the GSH oxidized by GPX, which correlates to the GPX activity in the sample. The reaction proceeds as:

$$ROOH + 2\ GSH \xrightarrow{GPX} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GRD} 2\ GSH + NADP^+$$

The supernatant obtained by cell extraction contains the cellular GPX protein. This sample is added to a solution containing GSH, glutathione reductase enzyme, and NADPH is added according to manufacturer's instructions. The assay conditions are at saturation, using 1 mM GSH, 0.2 mM NADPH, 0.22 mM tert-butyl hydroperoxide, and $\geq$0.4 U/ml glutahione reductase enzyme at pH 7.6, with the reaction run at 23-25° C in a total volume of 1.12 ml. Once the net rate of NADPH consumption is determined per minute, then the GPX activity can be calculated using 0.00622 as the molar extinction coefficient of NADPH. When the GPX activity is obtained, it is normalized to sample

DNA content for comparison between groups as the βAP toxicity adds a significant amount of protein which makes normalization to protein problematic.

3.8 Glutathione Reductase Enzyme Activity

Glutathione reductase (GRD) activity was assayed using a kit from Calbiochem. This activity is measured by measuring the rate of NADPH oxidation. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm and can be measured spectrophotometrically. The reaction proceeds according to the equation:

$$GSSG + NADPH + H^+ \xrightarrow{GRD} 2\,GSH + NADP^+$$

Rate limiting concentrations of GRD drive the dynamics, and the rate of absorbance decrease at 340 nm is directly proportional to the GRD in the sample, as the decrease in GSSG is equal to the consumption of NADPH. Assay conditions rely on saturating concentrations of NADPH (0.22 mM) and GSSG (1.0 mM) at pH 7.5 in a total volume of 1 ml using 200 µl of sample. The absorbance is followed for five minutes recording absorbance every 60 seconds. Once the net rate of NADPH consumption is obtained, GRD activity can be calculated using the molar extinction coefficient for NADPH (0.00622). Comparison among groups is done by normalization of activity to the DNA content of the sample.

3.9 DNA Content Assay

A simple procedure for assaying the DNA content of cells has been used by Labarca and Paigen [112] based on the ability of Hoescht dye 33258 to bind to double stranded DNA and enhance the fluorescence emitted at 492 nm (450) when the samples

are excited at 350 nm. A high salt buffer is used to dissociate the chromatin protein and minimize RNA interference. This allows the samples to be assayed without excessive manipulation and is sensitive to 10 ng/ml if necessary. After DNA pellets were obtained from cell extractions, the pellets are resuspended in the DNA buffer (0.05 M Na_2HPO_4/NaH_2PO_4 pH 7.4, 2.0 M NaCl, 0.002 M EDTA) and frozen at -80° C until assayed. When assays were performed, a working dye solution was made in the DNA buffer at a concentration of 1 µg/ml Hoechst dye 33258 (Sigma). The DNA samples were thawed and resuspended, sonicated for up to 30 sec, diluted 1:10 in the working dye solution and incubated for 3 h at room temperature in the dark. When ready, samples were read on a fluorometer at an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Standards were prepared from calf thymus DNA (Sigma), and the concentration of DNA determined by the absorbance at 260 nm where 50 µg/ml has an O.D. of 1.0. Standards diluted to working concentrations were sonicated for up to 30 sec and handled using the protocol established for samples. Standard curves were constructed and DNA content established using arbitrary fluorescence units graphed versus DNA concentration. Values were normalized to % of control.

3.10 Statistics

The significant treatment effects on cell viability, enzyme activity, and glutathione content were determined using ANOVA followed by Scheffe's post hoc test or Tukey's test, with significance determined at $p<0.05$. Comparisons between dose response relationships were made using two-way ANOVA to determine the significance of treatment presence or absence, with analysis by Tukey's test post hoc.

CHAPTER 4
LOW CONCENTRATIONS OF ESTRADIOL PROTECT AGAINST ßAP 25-35 INDUCED TOXICITY IN SK-N-SH NEUROBLASTOMA CELLS

4.1 Introduction

The genetic mutations reported to occur with some forms of familial AD have been linked with an overproduction of βAP from amyloid precursor protein [183], with an eleven amino acid fragment identified as the toxic portion of the molecule [222,223]. Mechanisms of cytotoxicity associated with βAP indicate that aggregation of the peptide [165,223] and contact with the plasma membrane are integral components [127], and further that mitochondrial degeneration is an early event [13]. Equivalent cytotoxic concentrations have been shown for both the full length peptide and the neurotoxic fragment in both rat primary neuronal cultures and B12 cell lines [14] and HT-22 cells [78].

Another consistently observed feature in AD patients is an asymmetric decline in brain glucose metabolism [89], which has been noted early in the course of disease [89,107,169,195]. The hypometabolism associated with these brain regions may account for the non-memory language and visuospatial function impairments seen in AD [89]. The idea that glucose utilization may play a role in memory processes has merit; retention of passive avoidance tasks and operant conditioning paradigms in rats is facilitated by post-training glucose administration [69,134]. Moreover, Craft et al. [40] have provided evidence that regulation of glucose may be a factor in AD pathology as blood glucose

levels in AD patients were shown to be elevated overall when compared to normal subjects. The role of glucose use in βAP toxicity has not been determined.

Estrogens could influence βAP toxicity by altering amyloid precursor protein (APP) processing, preventing the formation of βAP aggregates, or interfering with the effects of βAP fragments on neurons once fragments aggregate. Using a high estrogen receptor expressing human breast carcinoma cell line, Jaffe et al. [96] demonstrated that exposure to 2×10^{-9} E2 increased the amount of secreted, soluble form of APP, suggesting that estrogens may stimulate the non-amyloidogenic processing of APP. Alternatively, estrogens could prevent βAP toxicity by slowing the aggregation of βAP or by directly protecting neurons.

The SK-N-SH cell culture model is appropriate for the assessment of the neurotoxic effects of aggregated βAP. This cell line is derived from a human female subject, shows a neuronal phenotype, and has a doubling time of 5 to 7 days. The ease with which SK-N-SH cells can be cultured makes them very useful as a model in the assessment of neurotoxicity and neuroprotection. The present studies were undertaken to determine if the *in vitro* exposure to βAP caused cell death and whether simultaneous exposure to estrogens were neuroprotective. The prediction was that βAP causes an oxidative stress to cells, which could be attenuated by estrogens in their proposed mechanism as an antioxidant. The time course of βAP-induced toxicity was investigated, and additionally glucose utilization and lipid peroxidation were evaluated to assess the possible oxidative and metabolic stress components of this toxicity.

4.2 Materials and Methods

Toxicity assessment of βAP on SK-N-SH neuroblastoma cells was done at various concentrations of βAP. Experiments were initiated by lifting cells with 0.02% EDTA and plating at a density of 1 x 10^6 cells per well in appropriate treatment media. Cells were cultured in RPMI-1640 with 10% FBS with vehicle controls or supplemented with the addition of βAP (Bachem, Torrance, CA) at various concentrations. Lyophilized βAP 25-35 (1 mg) was initially dissolved in 200 µl dd H_2O then 800 µl PBS was added and diluted to a concentration range of 10-80 µM in RPMI-1640. Cells were incubated for 96 hours.

Time course studies of the acute effects of βAP, E2, and their combination on cell viability, glucose utilization and lactate production were performed at 3 h time points for a total of 15 h. Experiments were initiated by lifting cells with 0.02% EDTA and plating at a density of 1 x 10^6 cells per ml, allowing attachment overnight in RPMI-1640 media. Media was then replaced with appropriate treatment media. Cells were cultured in RPMI-1640 with 10% FBS and absolute ethanol (34 µM) as a vehicle control, or supplemented with the addition of βAP (Bachem, Torrance, CA), 17 ß-estradiol (E2; Steraloids, Wilton, NH), or βAP and E2 together.

Chronic studies were designed to assess the effects of βAP, E2, and their combination on cell viability and membrane oxidation over the course of 2 to 4 days. In these studies, experiments were initiated as described above and determinations were made at 48, 72 and 96 hours as indicated for TBAR production and cell viability.

The effects of pre-, co-, or post- estradiol treatment on ßAP induced toxicity were evaluated. Experiments were initiated by lifting cells with 0.02% EDTA and plating at a density of 1 x 10^6 cells per ml, allowing attachment in RPMI-1640 media. When attached, media was decanted and replaced with treatments for 24 h. At this time, initial treatment media was decanted and replaced with secondary treatment media for an additional 24 h. Pre-treated cells received E2 upon initiation of experiment and ßAP after 24 h of incubation. Co-treated cells received both E2 and ßAP upon initiation, and E2 after 24 hours of incubation. Post-treated cells received ßAP upon initiation and E2 after 24 h of incubation. ßAP treated cells received either vehicle control or ßAP upon initiation and the other after 24 h of incubation. E2 and vehicle treated controls received the appropriate treatment both at initiation and after 24 h of incubation. All cell counts were performed after 48 h of incubation using trypan blue exclusion as previously described.

Statistical analysis was performed between groups at each time point using ANOVA followed by Scheffe's post hoc tests. $p<0.05$ was considered to be significant. For each study, 3-5 wells per group were used.

4.3 Results

Four days of exposure of SK-N-SH cells to βAP 25-35 resulted in a dose-dependent loss of trypan blue excluding cells with cell numbers reduced by 36, 65, 70, and 83% at the 10, 20, 40, and 80 μM doses, respectively (Fig. 4.1). Because cells are plated at high density and are confluent in the wells, changes in cell number should reflect cell death rather than cell division, especially since the doubling time for SK-N-SH cells is 5 to

7 days. Examination of cells after βAP exposure showed shrinking of soma, fragmentation of neurites, and accumulation of cellular debris in the wells adding further support for this conclusion. The addition of 20 μM was used for subsequent studies.

Acute Toxicity Acute treatment with βAP reduced the number of trypan blue excluding cells by 5% at 3 h to a maximum of 35% at 15 h when compared to vehicle treated controls (Fig. 4.2A). Simultaneous exposure of cells to βAP and E2 (2 nM) resulted in a delay in the appearance of βAP-induced toxicity, as there was no change in cell number at 3 h when compared to vehicle-treated controls and only a 3% change at the 6 h time point. Additionally, E2 in combination with βAP reduced the severity of the insult as viable cell loss was decreased 40% relative to the βAP treated group at 15 h. E2 alone did not affect cell viability over the 15 h time course.

Chronic Toxicity Chronic βAP and E2 effects, assessed at the 48, 72, and 96 h time points (Fig. 4.2B), demonstrated a βAP induced cell loss of 70%, 65%, and 51% respectively, when compared to the vehicle controls (Fig 4.2B). Simultaneous E2 exposure decreased the cell loss attributed to βAP by 69%, 71%, and 56% respectively, at the 48, 72, and 96 h time points.

Lipid Peroxidation The effect of chronic exposure of cells to ßAP was examined for lipid peroxidation in the presence or absence of E2 (Fig. 4.3) as short term decreases in lipid peroxidation have been previously demonstrated when ßAP treated cells were pre-treated with high doses of E2 [16,71]. Increases in lipid peroxidation of 4.2 to 5.5 fold were observed with 48 h of ßAP treatment when compared to vehicle treated controls normalized to total cells or live cells, respectively (Fig. 4.3). Simultaneous E2 and ßAP

treatment decreased lipid peroxidation to 1.9 to 2.5 fold for normalization to total cells or live cells, respectively, at the 48 h time point (Fig. 4.3). At 72 h, ßAP treated cells showed a 0.75 fold increase in lipid peroxidation for total cells, while a 2-fold increase was seen when normalized to live cells. In cells treated with ßAP and E2, lipid peroxidation levels were not increased over the level of vehicle treated controls (Fig. 4.3). After 96 h of treatment with βAP, all cells were at the level of controls. Presumably, the lack of further increase in MDA in the βAP treated cells at this time reflects the completion of cell death in this group.

Glucose Utilization and Lactate Production Acute E2 treatment alone had no effect on glucose utilization over the 15 h time course; however, addition of ßAP increased glucose utilization by SK-N-SH cells by 25 and 44% when compared to controls at the 3 and 6 h time points, respectively (Fig. 4.4). At 9, 12, and 15 h of treatment, glucose utilization was significantly increased in the ßAP treatment group by 100, 72, and 76%, respectively, in comparison to vehicle treated controls (Fig. 4.4). E2 treatment with ßAP decreased glucose utilization at all time points, with reductions of 64% at 3 h to 83% at 15 h ($p<0.05$) (Fig 4.4). Acute effects on lactate production were similar (Fig 4.4) as ßAP treatment increased the amount of lactate detected in the media, contrasted by those treated with ßAP and E2, where lactate production was decreased by 57% at 15 h.

E2 Pre- and Post-Treatment Finally, the effects of pre-, co-, and post- E2 treatment on ßAP-induced toxicity were compared (Fig. 4.5). Cells pre-treated with E2 for 24 h, followed by 24 h of ßAP treatment showed a 17 % decrease in ßAP-induced toxicity when compared to cells treated with ßAP for the same time period (Fig. 4.5).

Further, toxicity to cells co-treated with E2 and ßAP was decreased by 38 % when compared to cells treated with ßAP for the same time period; however, E2 added to cells 24 h post-ßAP treatment showed no neuroprotective effect (Fig. 4.5).

4.4 Discussion

The present study provides evidence that low concentrations of E2 can attenuate ßAP-induced toxicity using either pre- or co-treatment. Further, co-treatment of E2 can reduce lipid peroxidation and decrease the metabolic load of the cell as demonstrated by a lowering in the rate of glucose utilization and lactate production. Behl et al. [16], as well as Goodman et al. [72], have demonstrated that micromolar concentrations of E2 reduce lipid peroxidation with 2 to 20 hours of steroid pre-treatment and an additional 20 hours of insult in HT-22 cells and primary hippocampal neurons. The findings here demonstrate that low doses of E2 protect SK-N-SH cells from βAP induced toxicity using both short and long term exposures. In addition, chronic low doses of E2 reduce lipid peroxidation.

There is much evidence supporting a decline in glucose metabolism early in AD [89]. Studies in humans using positron emission topography suggest that hypometabolism may be a component of AD pathogenesis, in as much as asymptomatic individuals at risk for AD show hypometabolism in brain regions that were reported to progress to pathology [107,169,195]. In addition, decreased concentrations of glucose transporters have been noted in brains of AD patients [193] and selective decreases in the expression of certain subunits of mitochondrial enzymes necessary for oxidative metabolism have been demonstrated [191]. Recently, mutations in mitochondrial DNA coding for cytochrome *c* oxidase have been linked to late-onset AD patients [44]. Consequently, the observation

that exposure of SK-N-SH cells to βAP increased glucose use in close association with cell death was surprising.

There are several plausible explanations for this paradoxical observation. The breakdown of the mitochondria, which has been reported to occur as early as two hours after treatment with ßAP [13] would result in marked reductions in ATP generation by oxidative metabolism. In order to meet the metabolic demand of the cell during an βAP-induced insult, an increase in glucose utilization for anaerobic metabolism would be the likely result. The observation of an acute increase in glucose utilization is consistent with a rapid βAP-induced mitochondrial compromise, as is the observed increase in the production of lactate. Alternatively, key lipid peroxidation repair mechanisms utilizing glutathione rely on the generation of NADPH [133]. NADPH is generated from the pentose phosphate pathway (PPP) at two places, the rate-limiting enzyme being glucose-6-phosphate dehydrogenase which uses glucose as its substrate, and 6-phosphogluconate dehydrogenase which acts on 6-phosphogluconate. With the observed increases in lipid peroxidation, glucose utilization would increase to allow for repair and maintenance of membrane integrity and it has been demonstrated that PPP activity is stimulated in response to H_2O_2 exposure in primary mixed cortical cultures [17]. Finally, the observed increase in glucose utilization with βAP exposure may represent the selective βAP toxicity of SK-N-SH cells that are metabolically less active. In this case, the cells more resistant to βAP would appear to utilize more glucose.

The neuroprotective effect of estrogens observed in response to βAP insult on cell viability may result from its ability to reduce membrane lipid peroxidation rather than its

mitogenic effects. The above data show no effects of estradiol on cell proliferation demonstrated by the lack of increase in live cell number in estrogen treated control cultures. Further, thymidine uptake in these cells is not affected up to 48 h of treatment in either a serum deprivation model or in the presence of serum [20]. Estrogens are reported to be potent antioxidants in a variety of *in vivo* and *in vitro* models [128,141,143,147,172,203] and high doses of this steroid have been shown to reduce lipid peroxidation caused by βAP [16,71] and this additionally correlates with cell protection. Further, estrogens reportedly reduce excitatory amino acid-induced neuronal toxicity [194], an insult that has an oxidative stress component [144], and a variety of non-estrogenic estratriene compounds have been shown to reduce neuronal cell death induced by serum deprivation [75] an insult that causes an apoptotic-type cell death [66]. Since both necrosis [13] and apoptosis [119] have been linked to ßAP induced toxicity and oxidative stress may participate in apoptotic signaling [57], the antioxidant activity of estrogens may largely contribute to their neuroprotective capacity.

In summary, βAP induced neuronal death is associated with membrane lipid peroxidation and a persistent hypermetabolism both of which are ameliorated by simultaneous exposure to low concentrations of 17 β-estradiol. The neuroprotection obtained with low doses of E2 may result from its antioxidant nature, and since many antioxidants are capable of regenerating or recycling with other compounds, may additionally depend on other compounds to demonstrate protection at physiologically relevant concentrations.

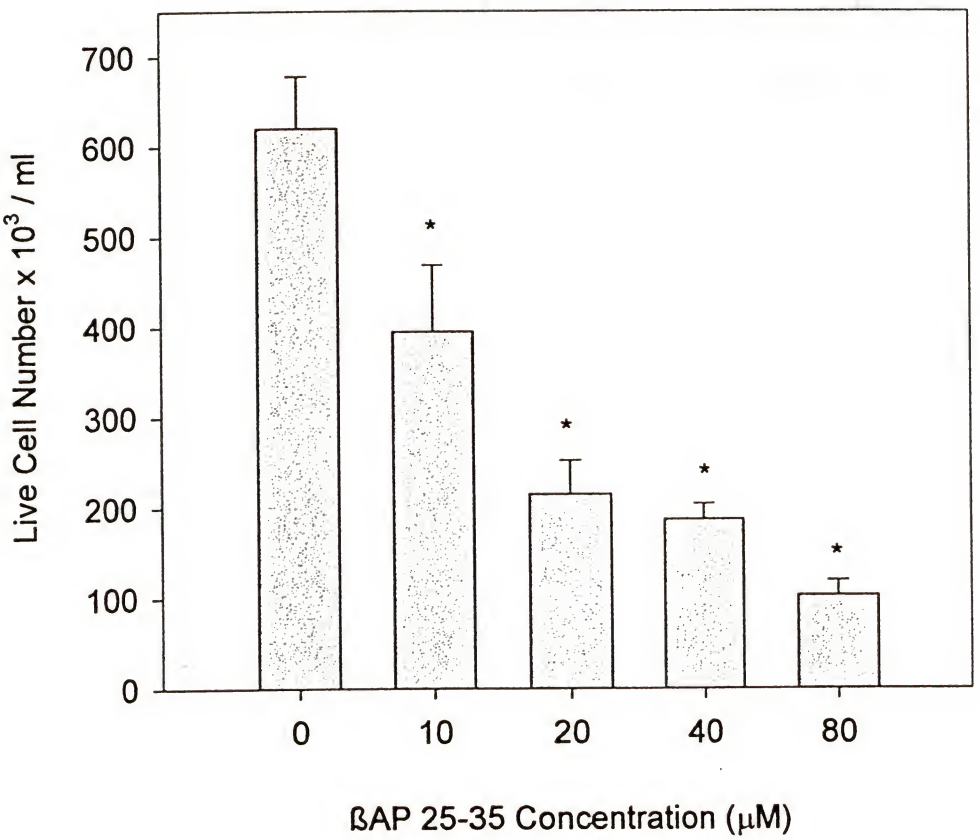

Fig. 4.1. Effects of dose of βAP 25-35 on SK-N-SH live cell number. Cells were plated at 1 x 106 cells per ml and were exposed to βAP 25-35 at the dose indicated. Four days later, the number of trypan blue excluding cells were determined. Depicted are mean ± SEM for n=4 wells. *$p<0.05$ versus the 0 (control) group.

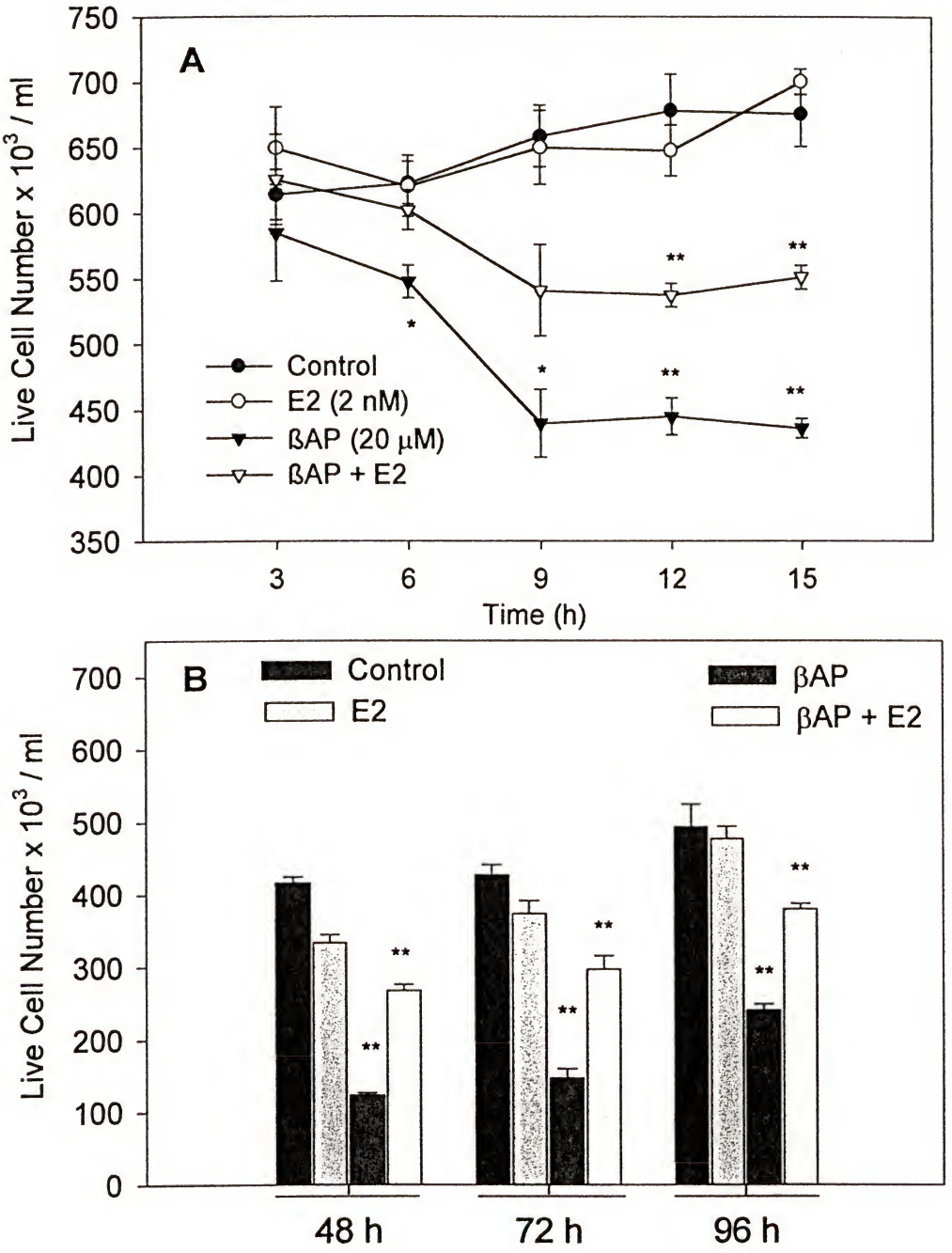

Fig. 4.2. Time course of βAP 25-35 treatment on SK-N-SH cells in the absence and presence of E2 (2 nM). A) Acute effects (3-15 h) and B) chronic effects (48-96 h). Live cell number determined by trypan blue exclusion with n = 4 wells per group. Represented are means ± SEM. *$p<0.05$ versus controls; **$p<0.05$ versus all other groups determined by ANOVA followed by Scheffe's test post hoc.

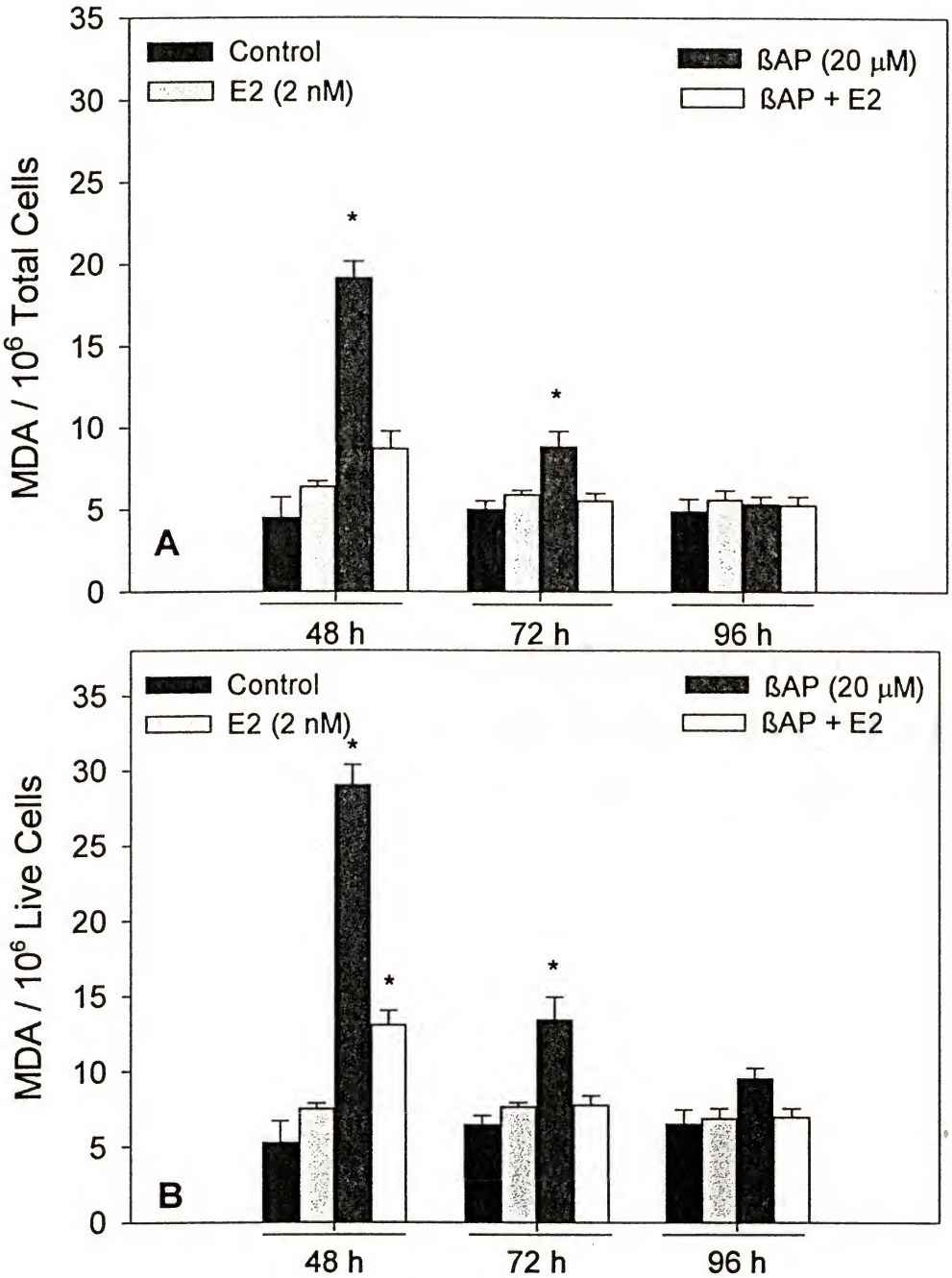

Fig. 4.3. Effects of treatment with βAP 25-35 (20 µM), E2 (2 nM), or their combination on lipid peroxidation in SK-N-SH cells. A) Total cells and B) Live cells. Lipid peroxidation expressed as malondialdehyde (MDA) produced per million cells. Represented are mean values ± SEM for n = 4 wells per group. *$p<0.05$ versus all other groups as determined by ANOVA followed by Scheffe's test post hoc.

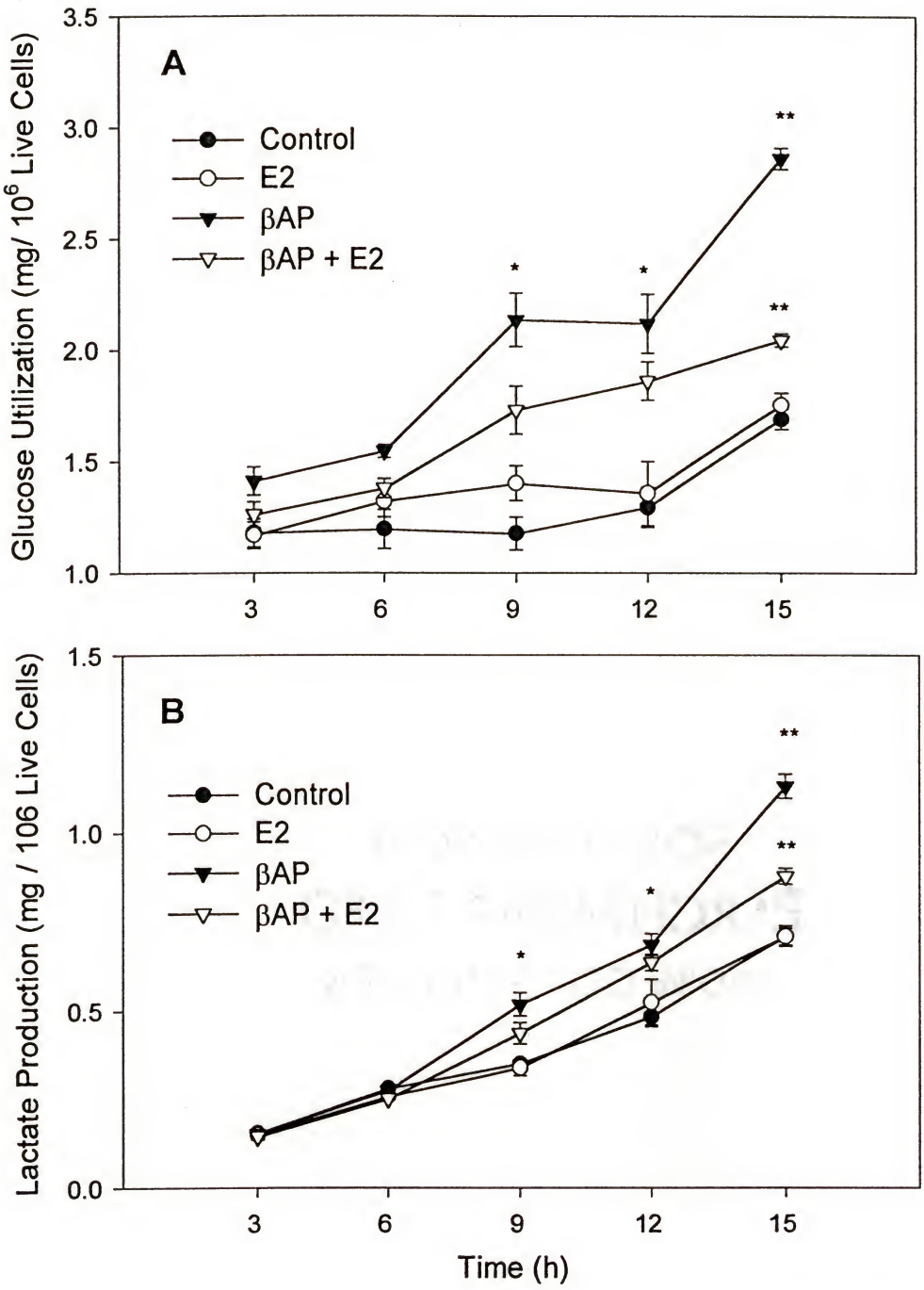

Fig. 4.4. Glucose utilization and lactate production in SK-N-SH cells subjected to βAP 25-35 treatment (20 μM), E2 (2 nM), or the combination, for the times indicated. A) Glucose utilization and B) Lactate production. Values obtained by subtracting media values after treatment from initial media values, and normalized per million live cells. Represented are means ± SEM unless too small to be depicted for n = 4 wells per group. *$p<0.05$ versus control groups; **$p<0.05$ versus all other groups as determined by ANOVA followed by Scheffe's test post hoc.

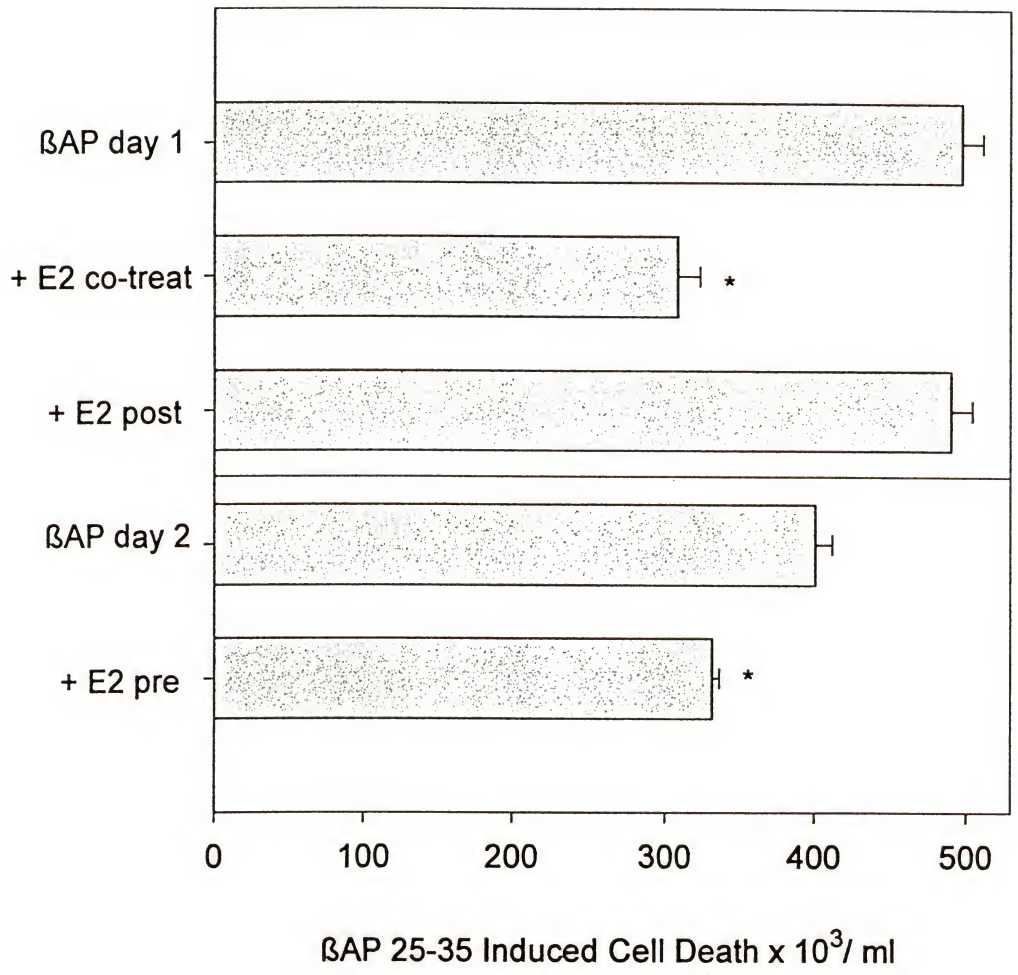

Fig. 4.5. Effects of E2 pre-, co-, or post-treatment on SK-N-SH cell death after 24 h or βAP treatment (20 μM). Cells were treated with E2 or βAP on either day 1 or 2, and assessed for trypan blue exclusion after a total of 48 h of treatment. Represented are mean values ± SEM for n = 4 wells per group. *$p<0.05$ versus all other groups as determined by ANOVA followed by Scheffe's test post hoc.

CHAPTER 5
IDENTIFICATION AND CHARACTERIZATION OF THE INTERACTION BETWEEN 17 ß-ESTRADIOL AND GLUTATHIONE FOR NEUROPROTECTION

5.1 Introduction

Antioxidants, i.e. any substance that under physiological conditions significantly delays or inhibits oxidation when present in low concentrations compared to those of an oxidizable substrate [87], are emerging as possible useful therapies for neurodegenerative conditions. Substances fitting this description and currently being used in clinical trials include vitamin E and estrogens. The demonstration that estrogens are capable of decreasing oxidative by-products at physiologically relevant concentrations *in vitro* links its neuroprotective ability to its antioxidant character. In addition, structure-activity relationship studies revealed that a phenolic A ring [15,76], a structural feature shared by many antioxidants, and at least three rings of the steroid structure [76] are required for estrogens to demonstrate neuroprotection.

The mechanism by which estrogen acts as an antioxidant at higher concentrations [16,71] may directly relate to its ability to scavenge free radicals [141]. I hypothesized that at physiologically relevant concentrations, estrogen may be participating in a cycle whereby estrogens are regenerated in a time-independent fashion by other endogenous antioxidants. Because reparation of lipid membranes relies on the glutathione peroxidase enzyme system [133], glutathione was targeted as a likely candidate for this interaction. The present studies were undertaken to investigate the possibility of an interaction

between 17 β-estradiol (E2) and glutathione in protecting cells against the toxicity of βAP 25-35 .

5.2 Materials and Methods

Materials Lyophilized βAP and E2 solutions were made as previously described. The estrogen receptor antagonist ICI 182,780 (Zeneca, Chesire, England) was dissolved in absolute ethanol and spiked into individual cell culture wells to obtain the 200 nM concentrations. α-Tocopherol acetate was initially dissolved in 200 µl absolute ethanol and diluted in cell culture media to the appropriate concentrations. Lipoic acid (thiotic acid), taurine (2-aminoethanoic acid), and ascorbic acid were initially dissolved in cell culture media and used at the concentrations indicated. Progesterone (Steraloids) was dissolved in absolute ethanol at 1 mg/ml and diluted in cell culture media and stored at 1 µM concentrations at -80° C. When needed, it was thawed and diluted to the indicated concentrations. Unless otherwise noted, materials were obtained from Sigma Chemical Corp.

SK-N-SH Neuroblastoma Cell Culture SK-N-SH cells used in the following experiments were in passes 28-46. Studies were initiated by plating 1 x 10^6 cells per well in 24 well plates, allowing attachment in regular media and then decanting that media and replacing with the appropriate treatment after 4 h. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) or RPMI-1640 without GSH (reduced) (Life Technologies, Grand Island, NY), supplemented with 10% FBS (except for serum deprivation experiments) and antibiotics, with absolute ethanol (34 µM) as a vehicle control, or supplemented with the addition of βAP 25-35 (20 µM), E2 (0.002 - 200 nM),

GSH (0.0325-325 μM), α-tocopherol acetate (50 μM), ascorbic acid (100 μM) , lipoic acid (10 μM), taurine (5 mM), ICI 182,780 (200 nM), progesterone (2-200 nM), or a combination as indicated. Selection of antioxidant concentrations were made on the basis of preliminary dose-response evaluations used to identify the maximal concentration at which neuroprotection was not obtained (data not shown). SK-N-SH cell viability was determined utilizing the trypan blue exclusion method after 48 h of incubation for serum deprivation experiments and 72 h of incubation for βAP 25-35.

Rat Primary Cortical Culture Treatments were made directly to primary cultures on culture day 10, maintaining a constant volume added regardless of treatment. Cultures were supplemented with the following treatments: Absolute ethanol (34 μM) and PBS as vehicle controls, βAP 25-35 (10 μM), E2 (0.02 nM-2 μM), GSH (3.25 μM), or combinations as indicated. The 10 μM concentration of βAP 25-35 was selected following preliminary studies aimed at causing a 40-60% cell death in 24 h. Once treatments were added, primary cultures were incubated for an additional 24 h and viability determined using the LIVE/DEAD® viability/cytotoxicity kit (Molecular Probes, Eugene, OR) according to manufacturer's instructions.

Statistics The significant treatment effects on cell viability were determined using ANOVA followed by Scheffe's post hoc test, with significance determined at $p<0.05$. Comparisons between dose-response relationships to determine the significance of GSH or E2 presence or absence were calculated using two-way ANOVA, and post hoc comparisons were performed using Tukey's Test.

5.3 Results

The neuroprotective capability of physiologically relevant concentrations of E2 relies on the presence of glutathione in the cell culture milieu (Fig. 5.1). The addition of βAP 25-35 to the culture milieu reduced the number of viable cells by 41% and 47%, respectively, utilizing DMEM media, which lacks GSH in the cell culture media, and RPMI-1640 manufactured specifically without GSH. Concomitant treatment with E2 (2 nM) had no effect (Fig. 5.1), which contrasted work from chapter 4 showing these same concentrations of E2 to be neuroprotective in RPMI-1640 media, which normally contains GSH. Based on the working hypothesis that GSH may play a role in this system, GSH (3.25 μM) was supplemented to cell culture media lacking GSH in the media. This addition of GSH was not neuroprotective alone, but when added with low concentrations of E2 (2 nM), it increased the number of viable cells by 88% and 47% in DMEM and RPMI-1640 lacking GSH, respectively (Fig. 5.1).

To further evaluate the interaction noted between E2 and GSH, the neuroprotective capability of GSH was investigated (Fig 5.2). In the absence of estrogen, high concentrations of GSH were necessary to achieve neuroprotection, with a significant reduction in βAP 25-35- induced toxicity of 50% at the 325 μM dose. By contrast, in the presence of E2 (2 nM), the neuroprotective dose of GSH was reduced to 0.325 μM. Two way ANOVA revealed a highly significant effect of E2 on the GSH induced neuroprotection ($F: 41.4$; $p<0.001$).

Utilizing this information, we analyzed the ability of E2 to increase the number of viable cells exposed to βAP 25-35 (Fig 5.3). In the absence of GSH, high concentrations

of E2 (200 nM) were necessary for significant neuroprotection (Fig 5.3). However, in the presence of a non-protective dose of GSH (3.25 μM), the neuroprotective dose of E2 shifted from 200 nM to 0.2 nM (Fig 5.3). Again, two way ANOVA revealed a highly significant effect of the presence of GSH on the neuroprotective effect of E2 (F: 44.33; $p<0.001$).

To ensure that this synergy was not due simply to cell origin or tumorigenicity, similar experiments were performed in rat primary cortical neurons. Again, the ability of E2 to protect neurons was evaluated in the presence and absence of GSH (3.25 μM) (Fig. 5.4). The addition of βAP 25-35 (10 μM) to primary cortical neurons resulted in a 39% to 40% reduction in the average number of viable cells per field in the absence and presence of GSH, respectively (Fig. 5.4). When increasing concentrations of E2 were evaluated against βAP 25-35, 200 nM E2 was the lowest concentration found to be protective (Fig 5.4), which is in full agreement with the SK-N-SH cell line studies (Fig. 5.3). With the addition of GSH (3.25 μM), all concentrations of E2 of 2 nM or higher were neuroprotective (Fig. 5.4). Evaluation of the effect of GSH on the neuroprotective action of E2 in rat primary cultures using two-way ANOVA demonstrated a significant interaction (F:8.53; $p<0.005$).

The specificity of this estrogen-antioxidant interaction for the GSH system was evaluated using the SK-N-SH cell model system with a concentration of E2 (20 nM) near the protective threshold of estrogen alone in the presence or absence of a previously determined non-protective dose of several other well known antioxidants (Table 5-1). In four separate experiments, the reduction in viable cells ranged from 35% to 68% when

subjected to a βAP 25-35 (20 μM) challenge. The 20 nM dose of E2 alone was either non-protective or slightly protective (Table 5-1). No significant effect was observed on βAP 25-35 induced toxicity with the addition ascorbic acid (100 μM), α-tocopherol acetate (50 μM), taurine (5 mM), or lipoic acid (10 μM) alone or in combination with E2 (Table 5-1). However, the addition of GSH (3.25 μM) or the oxidized form of glutathione (GSSG, 1.5 μM) was enough to significantly reduce the cytotoxicity of βAP 25-35 when 20 nM E2 was present (Table 5-1).

The insult specificity for the E2-GSH interaction was also evaluated using the SK-N-SH cell line, this time using serum deprivation as the insult (Fig. 5.5). In the absence of serum, 48 h of incubation resulted in a 49% decrease in live cell number, which was not affected by the presence of GSH (3.25 μM) alone. This is contrasted by the significant impact that the presence of GSH had on the protection afforded by estrogens ($F=19.488$; $p<0.001$) using this insult. In the absence of GSH, E2 protection was only significant at the 2 μM dose (97 %), while in its presence, 2 nM E2 demonstrated significant protection (71%).

The importance of an estrogen receptor in this interaction was determined by using ICI 182,780 (ICI), as an estrogen receptor antagonist (Fig. 5.6). Again, βAP 25-35 (20 μM) was added to SK-N-SH cells in the presence and absence of E2 (2 nM), GSH (3.25 μM), GSH plus E2, and/or ICI (200 nM). βAP 25-35 reduced viable cells after 72 h of exposure by 54% when compared to vehicle controls. Using concentrations of E2 with GSH that were not protective when administered alone but were neuroprotective when added together, the addition of ICI in 100-fold excess of the E2 concentration did not

significantly alter the protective effects of E2 and GSH in combination (Fig. 5.6). Indeed, ICI addition alone exerted neuroprotective activity.

Finally, the effects of progesterone on this system were evaluated (Fig. 5.7). In the presence of 200 nM progesterone, no significant impact was noted on the toxicity of the βAP 25-35 insult, which in this experiment was a 46% decrease in live cell number (Fig. 5.7). However, the presence of progesterone did have a significant impact on the interaction between E2 and GSH in protecting cells against βAP 25-35 toxicity. In the absence of progesterone, the E2/GSH increase in live cell number was 56%. With the addition of either 2 or 200 nM progesterone, the amelioration of toxicity exhibited by the E2/GSH combination was antagonized.

5.3 Discussion

A novel synergistic interaction between E2 and glutathione for neuroprotection has been identified. This interaction is independent of species origin and the tumorigenicity of the cells, as we have demonstrated protection in a human neuroblastoma cell line, in rat primary cortical neurons, and the HT-22 transformed mouse hippocampal cell line [78]. Further, this affect appears to be independent of the type of cytotoxic insult used as serum deprivation demonstrates the same synergy as βAP toxicity in SK-N-SH cells, and independent of the form of βAP used as synergistic interactions were noted against both βAP 1-40 and βAP 25-35 in HT-22 cells [78]. While differences exist in the glutathione-induced shift in the neuroprotective potency of E2 in these cell types, these may directly relate to the different cell culture techniques and the methods used to assess viability. In either case, we can resolve discrepancies with regards to differences reported between this

work and others [16,71] for the neuroprotective concentrations of estrogens. In the previous chapter, a cell culture milieu containing GSH was used, while others used media where GSH was absent.

Mounting evidence supports the hypothesis that the neuroprotective activity of estrogens resides in their antioxidant capacity. Decreases in oxidative by-products are correlated with decreases in cellular toxicity in my previous studies [79], as well as those done by others [15,71]. Since the actions of most antioxidants are multifaceted, estrogens may be working through several mechanisms to provide neuroprotection. Estrogen can participate in the non-enzymatic reduction of free radicals [143], as demonstrated in a cell free system where peroxy-nitrite radicals were found to be reduced by estrogens [141] at the same concentrations in which neuroprotection was observed in the absence of glutathione. Further, estrogens can participate in iron reduction [175], which may be paramount to decreasing the production of free radicals. Multiple studies have demonstrated that estrogens decrease lipid peroxidation in a variety of model systems [15,16,71,79,106,113,203,205]. Estradiol has also been shown to reduce oxidative impairment of membrane transporters for ions and glucose resulting from βAP 25-35 exposure [106]. In addition, estrogens did not prevent impairment of membrane transport systems from toxic lipid peroxidation by-products [106] providing additional support for the idea that their antioxidant nature is the basis for their activity in neuroprotection.

GSH also exerts its antioxidant activity through several mechanisms [133]. GSH can scavenge free radicals via a non-enzymatic mechanism [133]. In our system, high concentrations of GSH (325 μM) were necessary to protect cells from the βAP insult (20

μM). This neuroprotective concentration is a much higher concentration of GSH than is present in extracellular fluids as the intracellular level of GSH in mammalian cells is 0.5-10 mM, whereas micromolar concentrations are typically found in blood plasma and extracellular environments [133,196]. Perhaps a more practical explanation involves the ability of GSH to act on intracellular peroxides via GSH peroxidases and GSH S-transferases [133]. This system functions in the defense against free radicals through the reduction of hydrogen peroxide. This action of GSH may be relevant to the βAP insult, since generation of increased amounts of H_2O_2 has been demonstrated in both βAP 25-35 and βAP 1-40 induced toxicity [14] and increases in the activity of glutathione peroxidase are correlated with increases in cell survivability for both peptides [177].

The specificity of estrogen's interaction for this glutathione system is supported by two lines of evidence. First, there are no apparent interactions noted between estrogen and the other sulfur containing compounds tested, lipoic acid or taurine, or any other antioxidants, including ascorbic acid or α-tocopherol. It is interesting to note that while α-tocopherol is a powerful antioxidant in its own right, estrogen has been argued to be even more powerful [203], postulating that this may be due to the ability of the tocopheroxyl radical to regenerate estrogen. No such interactions were observed in this system. Second, the ability of oxidized glutathione to work with higher concentrations of estrogens indicates that they may be interacting in the glutathione peroxidase/reductase process. *In vivo* evidence supports this idea as oral contraceptive use has been correlated with an increase in glutathione peroxidase activity [30,125].

The observed synergistic interaction between E2 and GSH may be mediated through a mechanism independent from a classical estrogen-receptor. The use of ICI as an antiestrogen is support for this idea, yet interpreting this data is difficult. The amount of the ICI compound used, which in 100-fold excess of the E2 concentration satisfies the criteria required for demonstration of competitive inhibition, is at concentrations that other phenolic A ring containing compounds have demonstrated protection in the absence of glutathione [78]. Likewise, effects of the ICI compound unrelated to blocking E2 receptors cannot be ruled out. It would be interesting to determine if the ICI compound were capable of interacting with GSH at non protective doses for the compound, but other studies with this compound had higher priority and supplies of the compound were limited. The physiological relevance for the E2/GSH interaction is supported by the ability of progesterone to oppose their combined effects.

In closing, perhaps increasing the extracellular content of GSH is the necessary component for neuroprotection with estrogens. At the very least, the observation that the potency of E2 was markedly affected by physiological concentrations of GSH in the culture environment indicates that careful consideration for antioxidant chemistry must accompany experimental design when assessing antioxidant drugs *in vitro*.

Table 5-1. Effects of 17 ß-estradiol (E2), a variety of antioxidant treatments (TRT), and their combination on the βAP 25-35 induced toxicity in SK-N-SH cells, with the number of trypan blue excluding cells expressed as live cell number x 10^3 / ml for n= 3-4 wells per group after 72 h of treatment.

	Ascorbic Acid (100 μM)	α-Tocopherol (50 μM)	Taurine (5 mM)	Lipoic Acid (10 μM)	GSH (3.25 μM)	GSSG (1.5 μM)
Control	365±9	641±25	628±13	628±13	657±30	657±30
βAP	118±9*	366±24*	408±10*	408±10*	331±17*	331±17*
βAP + E2	144±9*	407±21*	394±3*	393±3*	357±19*	357±19*
βAP + TRT	124±6*	448±12*	402±9*	406±7*	369±7*	350±29*
βAP + E2 +TRT	188±11*	456±14*	439±11*	416±10*	534±16♦	513±7*♦

*= $p<0.05$ versus vehicle treated controls
♦=$p<0.05$ versus βAP treated controls

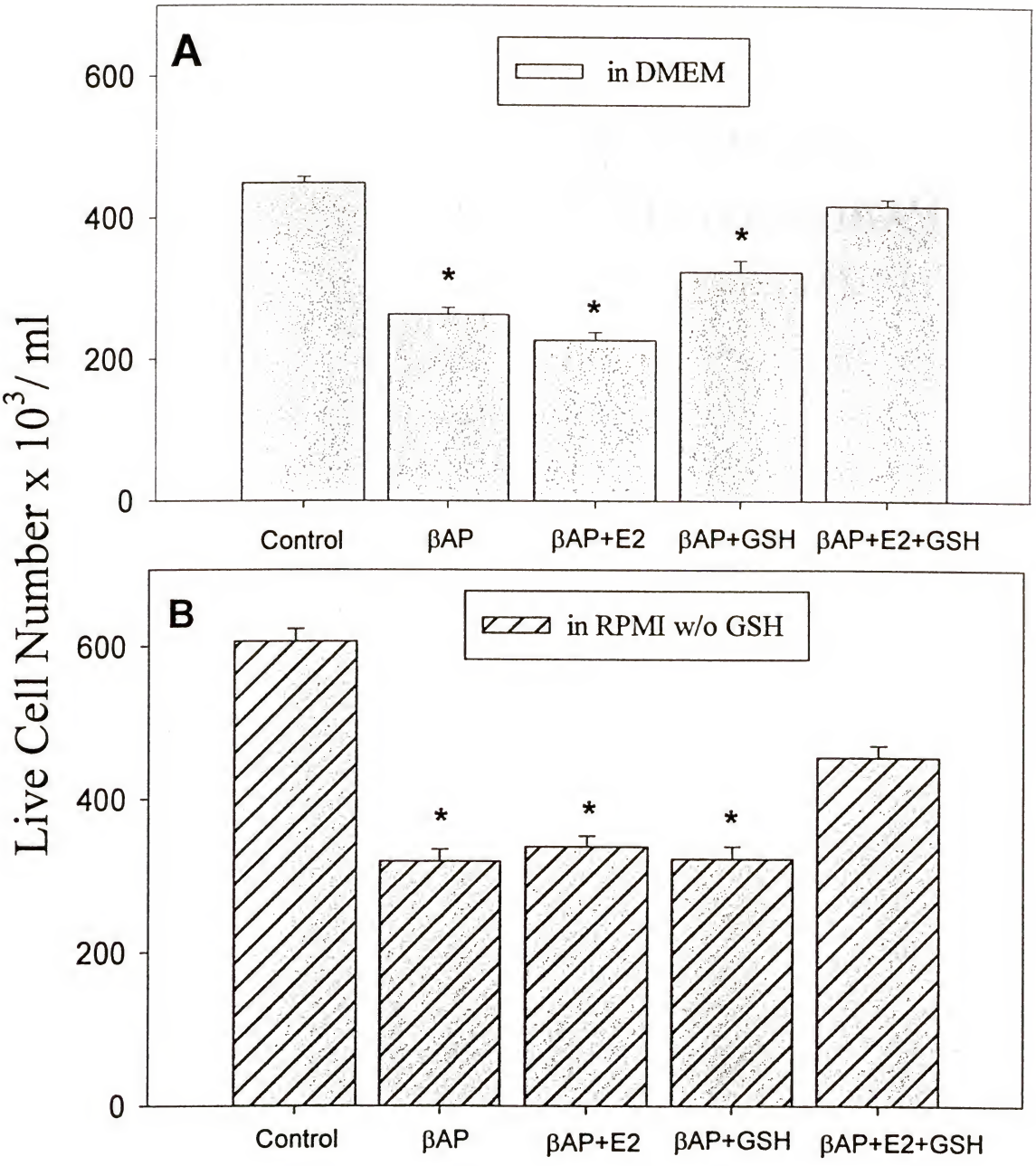

Fig. 5.1. Effects of E2 (2 nM) and GSH (3.25 μM) on the toxicity induced by βAP 25-35 (20 μM) in different cell culture media. A) in DMEM and B) in RPMI w/o GSH. Cells were plated at 10^6 cells/well and exposed to treatments as indicated for 72 h. Controls represent mean of all groups not exposed to βAP. Depicted are mean values ± SEM for n = 4-5 wells per group. *$p<0.05$ versus controls determined by ANOVA followed by Scheffe's test post hoc.

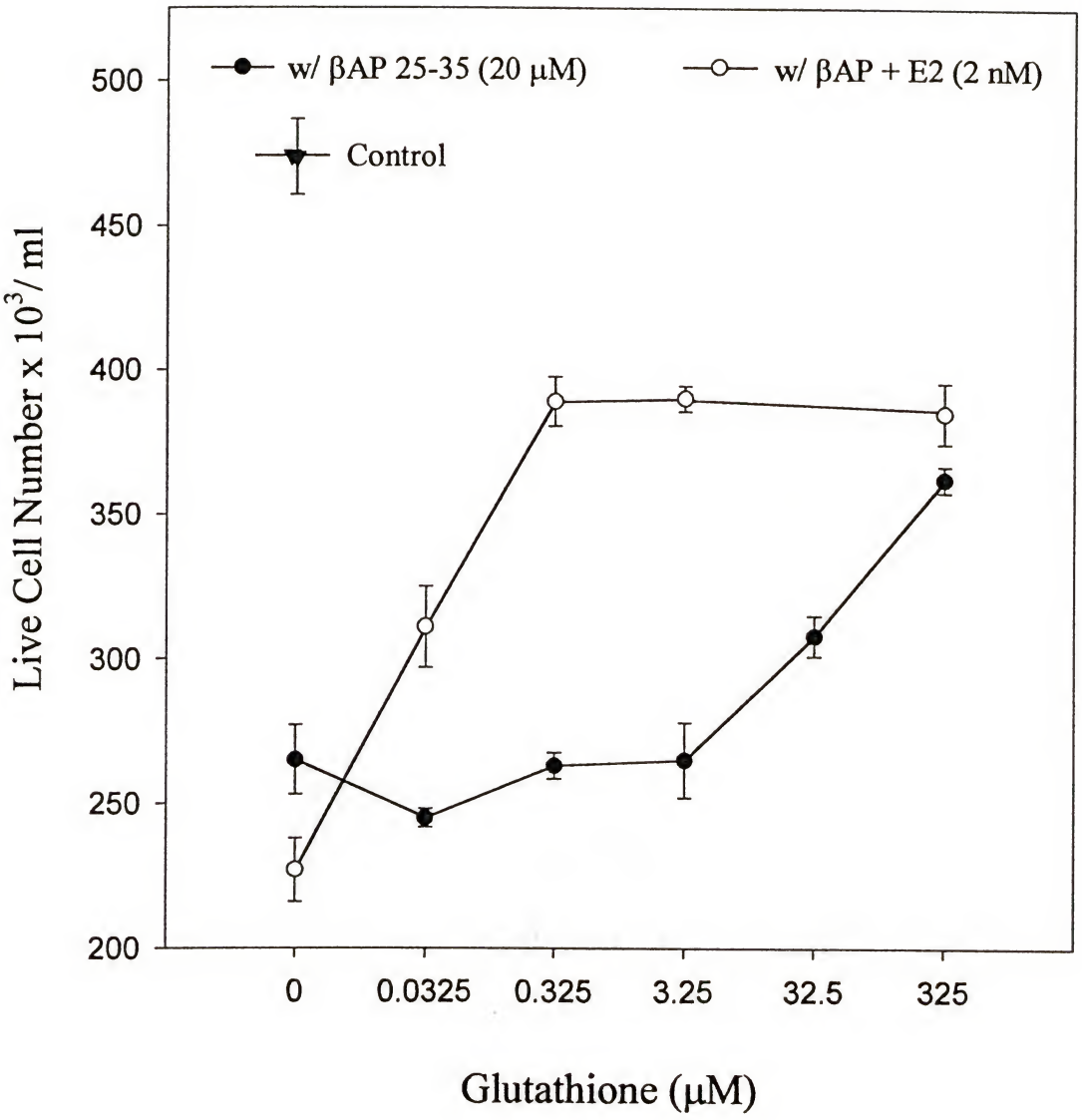

Fig. 5.2. Effects of βAP 25-35 on SK-N-SH live cell number at various doses of GSH in the presence and absence of a non-protective dose of E2 (2 nM). Cells were plated at 10^6 cells/well and subjected to the treatments indicated for 72 h. Controls represent groups not exposed to βAP, pooled together after it was determined they were not different from each other. Depicted are mean values ± SEM for 6 wells per group. The effect of E2 on GSH was highly significant ($F=41.48$; $p<0.001$).

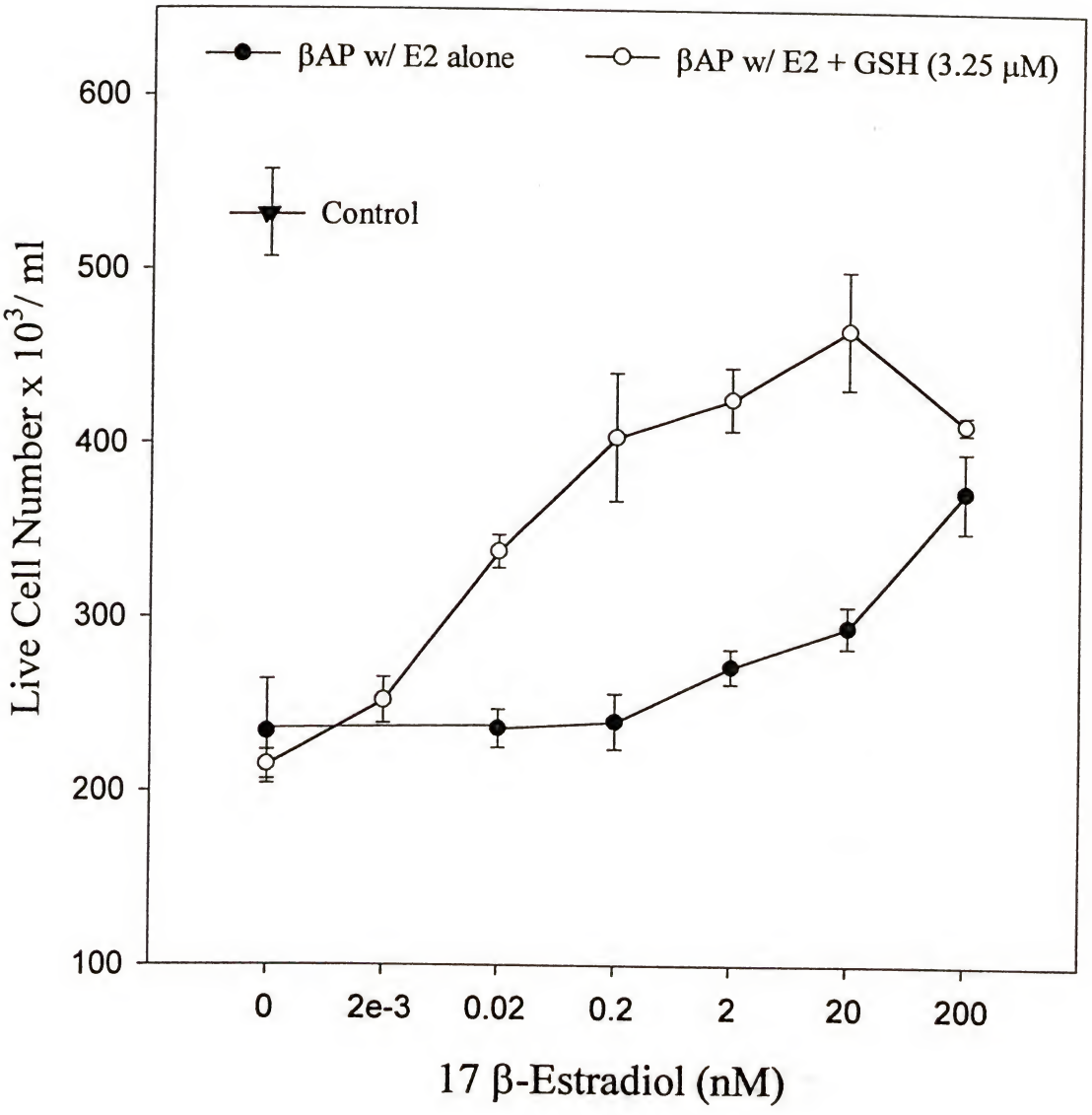

Fig. 5.3. Effects of βAP 25-35 (20 μM) on SK-N-SH live cell number at various doses of E2 in the presence and absence of a non-protective dose of GSH (3.25 μM). Cells were plated at 10^6 cells/well and subjected to the treatments indicated for 72 h. Controls represent all groups not exposed to βAP after it was determined they were not different from each other. Depicted are mean values ± SEM for 4 wells per group. The effect of GSH on the E2 response was highly significant ($F=44.33$; $p<0.01$).

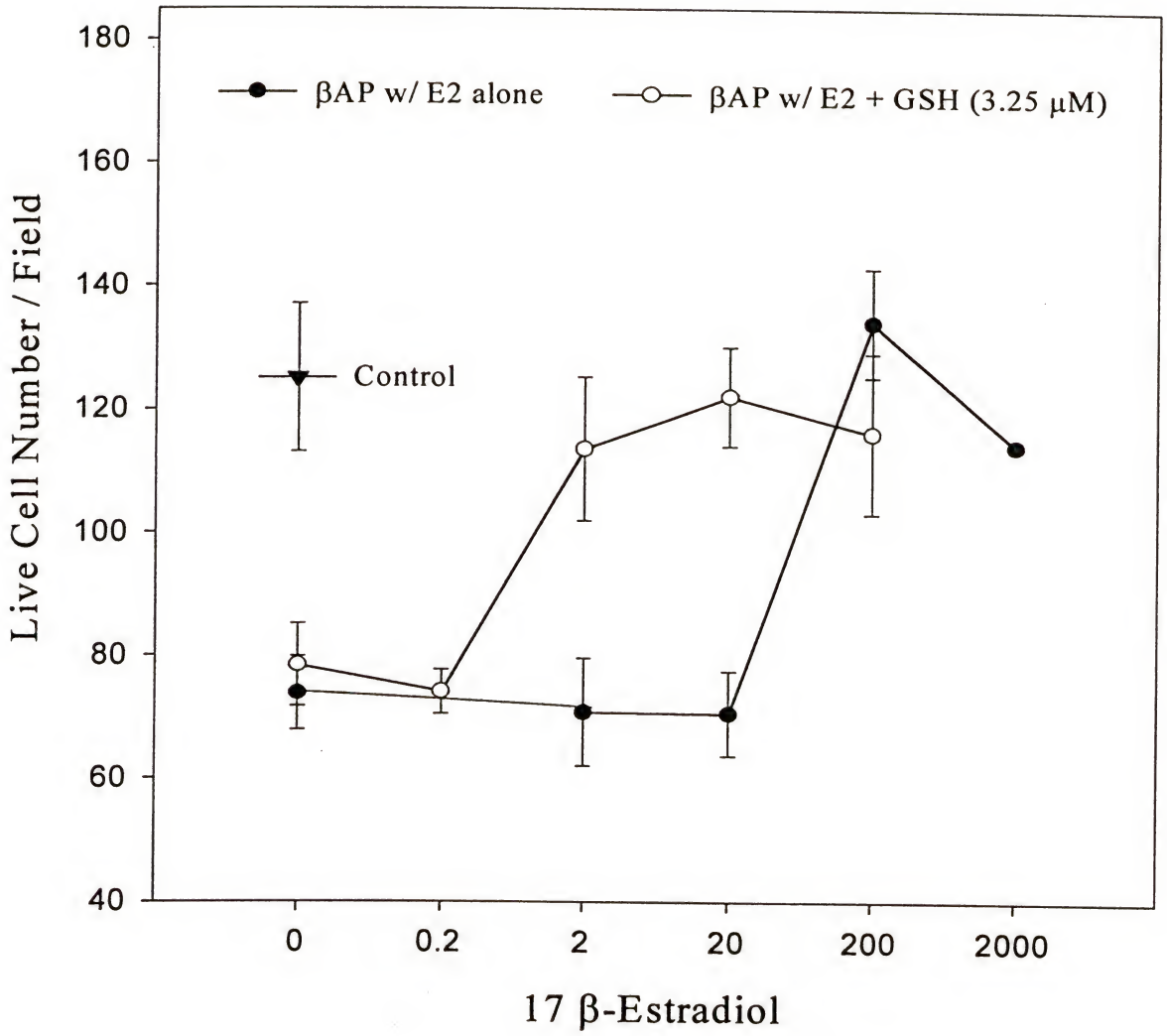

Fig. 5.4. Effects of βAP 25-35 (10 μM) on the live cell number in rat primary cortical neurons per photographic field at various concentrations of E2 in the presence and absence of GSH (3.25 μM). Cells were exposed to the treatments indicated on culture day 10 for 24 h. Depicted are mean values ± SEM for 4-7 plates per group, except for controls, which is an average of all groups not exposed to βAP. The effect of GSH on the E2 response was highly significant ($F=8.53$; $p<0.005$).

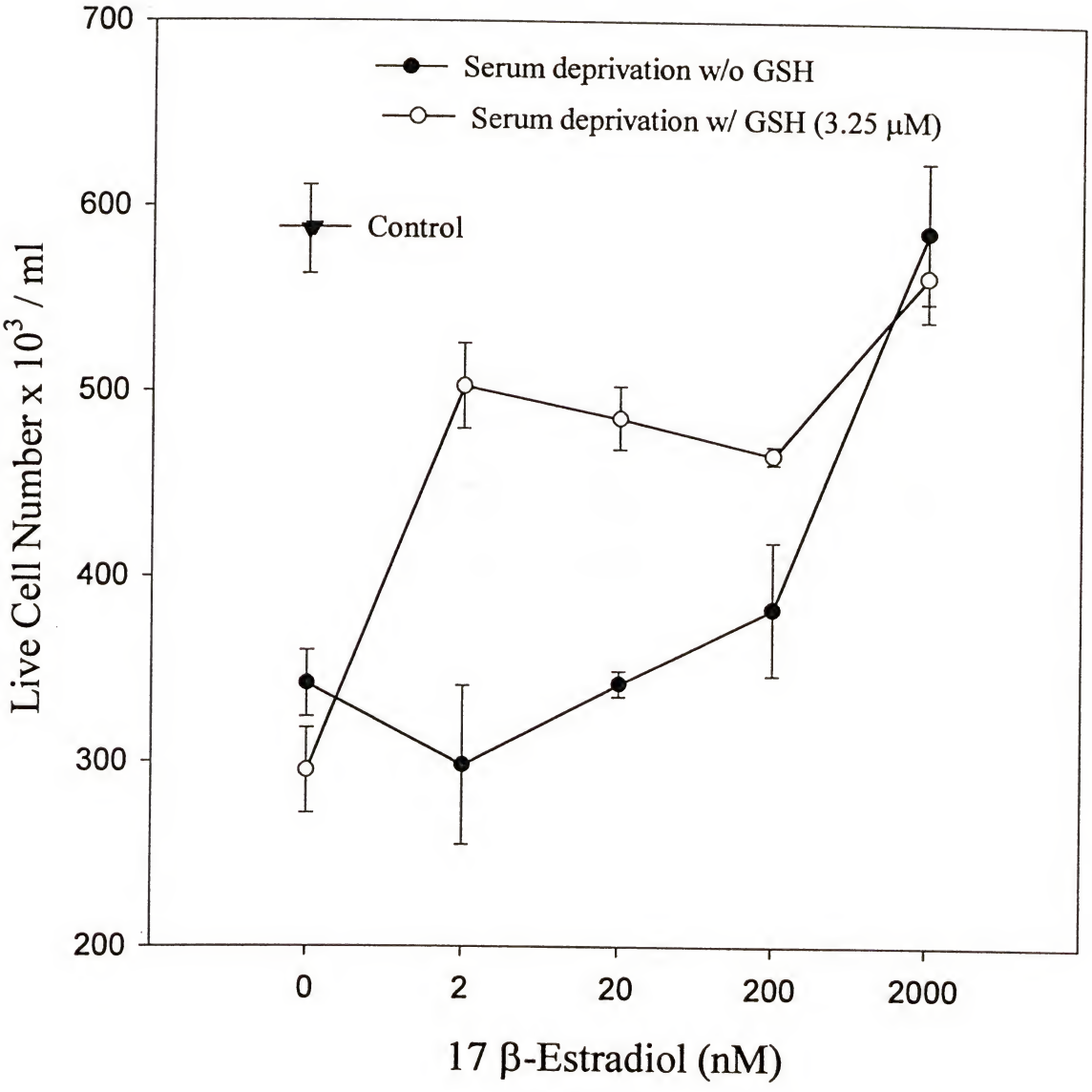

Fig. 5.5 Effects of serum deprivation on the live cell number in SK-N-SH cells at various concentrations of estrogen in the presence and absence of GSH. Cells were plated at 1 x 10^6 cells/well and exposed to serum deprivation and the treatments indicated for 48 h. Depicted are mean values ± SEM for 3-4 wells/group, except for controls, which is an average of all groups not exposed to serum deprivation. The effect of GSH on the E2 response was highly significant ($F=19.488$; $p<0.001$).

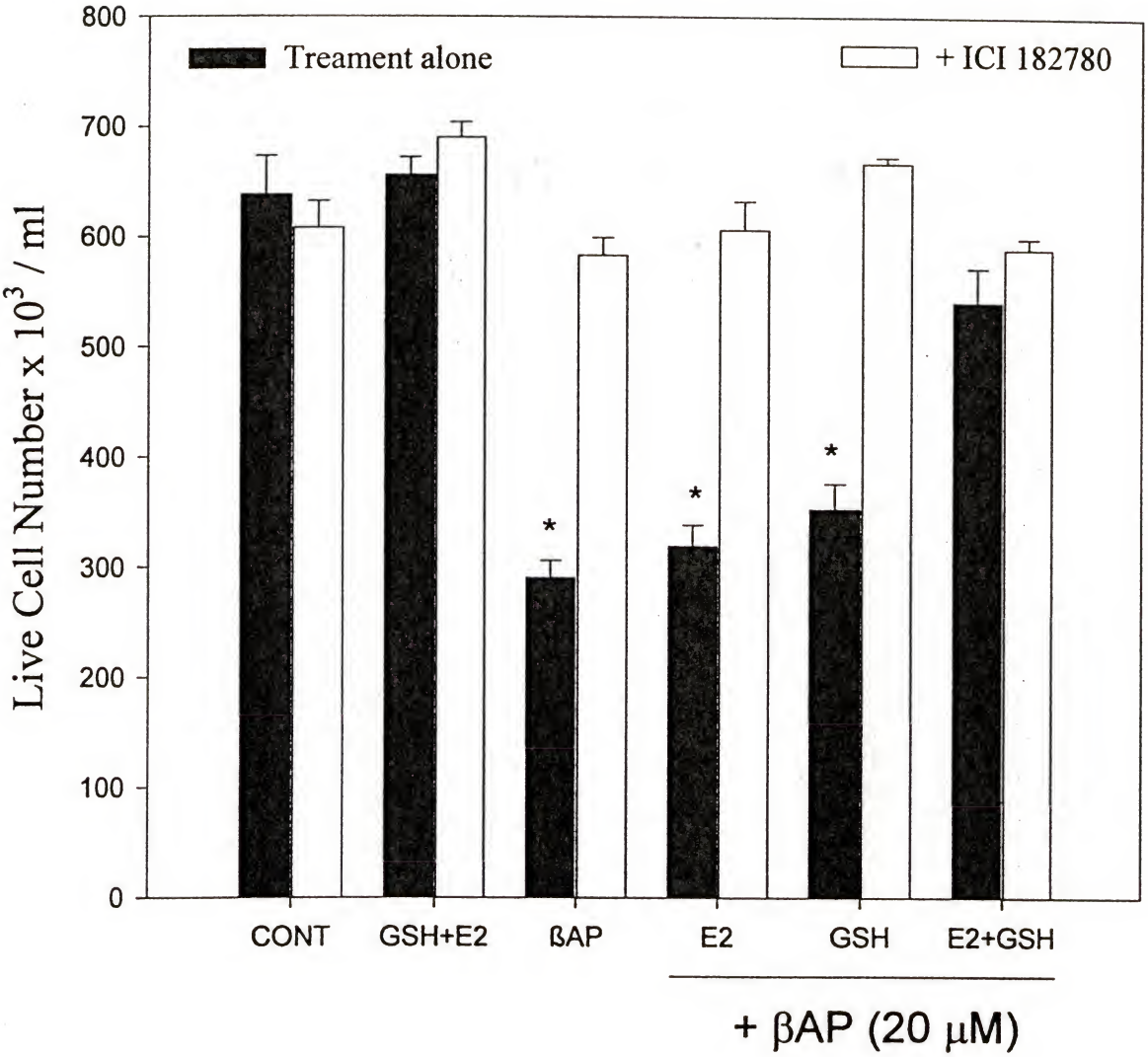

Fig. 5.6. Effects of antiestrogen ICI 182,780 (ICI, 200 nM) on βAP 25-35 (20 μM) induced toxicity in SK-N-SH cells. Cells were exposed to doses of E2 (2 nM) and GSH (3.25 μM) which alone are not protective. Cells were plated at 10^6 cells/ml and exposed to vehicle or βAP with E2, GSH, or E2+GSH as indicated for 72 h in the presence or absence of ICI. Depicted are the mean values ± SEM for 3 wells per group. *$p<0.05$ versus controls determined by ANOVA followed by Scheffe's test post hoc.

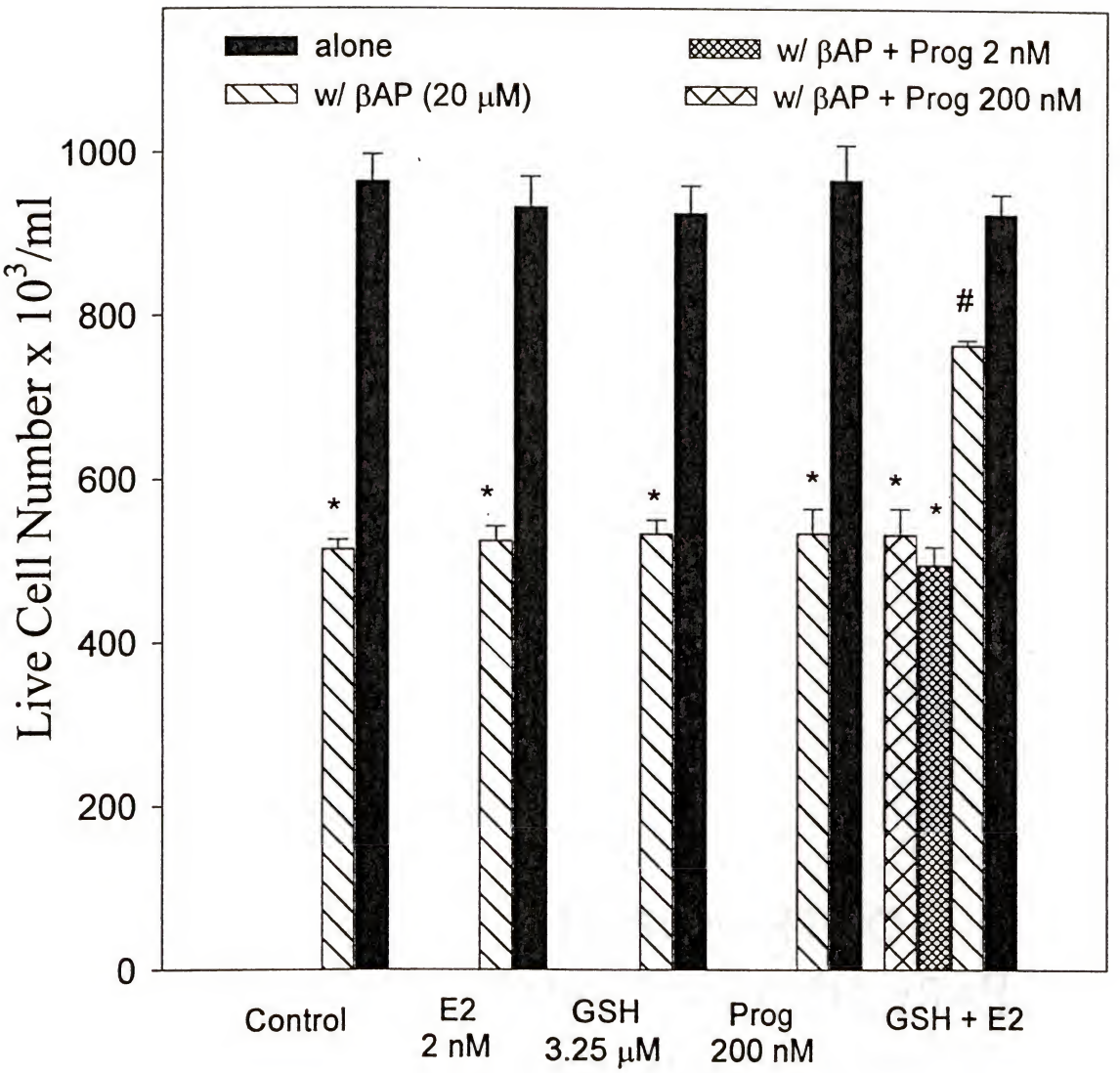

Fig. 7. Effects of progesterone on the neuroprotection obtained from the combined treatment of E2 and GSH on SK-N-SH cells subjected to βAP 25-35 induced toxicity. Cells were plated at 10^6 cells/well and incubated with the indicated treatments for 72 h. Depicted are mean values ± SEM for 4 wells/group. *$p<0.05$ versus controls, #$p<0.05$ versus all other groups as determined by ANOVA followed by Tukey's test post hoc.

CHAPTER 6
ZINC AND ZINC/GLUTAMATE NEUROTOXICITY IN SK-N-SH NEUROBLASTOMA CELLS AND PROTECTION AFFORDED BY 17 ß-ESTRADIOL

6.1 Introduction

Decreases in the mortality associated with stroke related events have been observed in females taking estrogen therapy (for review see [156]). In addition, estrogen administration to rats either concomitantly or before middle cerebral artery occlusion result in a reduction in mortality and ischemic damage/lesion size [192,224]. When estrogen is given after focal ischemia, protection is noted in the penumbra region, suggesting a protective role for estrogen in the excitotoxicity phase of the insult [224]. Several groups have substantiated that excitotoxic events contribute to stroke related pathophysiology and that neurons exposed to high amounts of glutamate are at an increased risk for cell death [36]. Recent evidence indicates that toxic concentrations of zinc released during ischemia/repurfusion may be contributing to excitotoxicity and producing neuronal damage [37]. Co-localization of zinc with glutamate in synaptic vesicles [163] highlights the possibility that excitotoxicity and neuronal death may involve zinc, glutamate or the combination. The hypothesis governing this work is that one component of estrogen's protection in stroke may reside in its ability to protect against zinc neurotoxicity or a combination of zinc and glutamate toxicity.

6.2 Materials and Methods

Materials 17 β-estradiol (E2; Steraloids, Wilton, NH), was initially dissolved at 10 mg/ml in absolute ethanol Fisher Scientific Inc., Orlando, FL) and diluted in media to obtain the necessary concentrations. Zinc Chloride (Sigma Chemical Corp., St. Louis, MO) was initially dissolved in 10 ml ddH_2O and diluted in cell culture media to the appropriate concentrations. Glutathione (GSH; Sigma) and l-glutamic acid (Sigma) were dissolved in cell culture media and diluted to the appropriate concentrations. (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA; Sigma) N-methyl-D-aspartatic acid (NMDA; Sigma) 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris, Ballwin, MO) in the water soluble hydrate form, were dissolved in ddH_2O and diluted to the appropriate concentrations in cell culture media.

Methods SK-N-SH cells used were obtained from American Type Tissue Collection at pass 31 and used in passes 48-60. Cells were plated at 1×10^6 cells/ well in DMEM and allowed to attach for 2-4 h. At this time, media was decanted and replaced with the appropriate treatment in DMEM, using absolute ethanol (34 μM) as a vehicle control, and/or supplemented with the addition of Zn^{2+} (50-400 μM), E2 (0.002 - 200 nM), GSH (3.25 μM), CNQX (500 μM), glutamate (0.1-10 mM), AMPA (500 μM), NMDA (500 μM), or KA (500 μM), or a combination as indicated. AMPA, NMDA and KA are glutamate receptor agonists, while CNQX is a glutamate receptor antagonist. The K_m reported for the glutamate receptor in cultured neurons is 33-40 μM [55], and the choice for the 500 μM dose for CNQX is in at least 10 fold excess. After up to 20 h of treatment, SK-N-SH cell viability was determined utilizing the trypan blue exclusion

method . All suspensions were counted on a Neubauer hemacytometer and two counts of live and dead cells were made for each aliquot.

6.3 Results

SK-N-SH neuroblastoma cells subjected to increasing concentrations of Zn^{2+} showed significant toxicity at the 200 μM dose (Fig. 6.1) resulting in cell death of 32% when compared to controls, and an LD_{50} of 248 ± 24.1 μM (Fig. 6.1). A short time course of toxicity using the 200 μM dose of Zn^{2+} revealed significant cell death of 30% at the 3 h time point (Fig. 6.2) when compared to vehicle treated controls.

Next, the ability of 17 β-estradiol to protect SK-N-SH cells against zinc toxicity was investigated (Fig. 6.3). The toxicity attributed to Zn^{2+} when compared to controls in this experiment was 27% (Fig. 6.3). Concomitant treatment with increasing doses of E2 resulted in reduced cell death. Forty percent fewer cells were killed by the addition of Zn^{2+} at the 200 nM dose of estrogen (Fig. 6.3). Because of the similarities between these results and experiments from the previous chapter, synergy between estrogen and glutathione was evaluated. In the presence of GSH, the protective dose of estrogen decreased 100-fold, with the 2 nM dose of E2 resulting in a 72% increase in live cell number. The contribution of GSH to E2 protection was found to be significant using two way analysis of variance for comparison ($F=4.244$; $p=0.002$).

Given that Zn^{2+} toxicity may be a component of excitotoxicity, the effects of glutamate on non-toxic doses of Zn^{2+} were explored. It is interesting to note that SK-N-SH cells are refractory to large doses of glutamate (10 mM) alone (Fig. 6.4); however, glutamate (10 mM) in the presence of non-toxic doses of Zn^{2+} resulted in a significant

decrease in live cell number of 31% at the 100 μM dose of Zn^{2+} and a decrease of 40% at the 50 μM dose (Fig. 6.4). Using the 100 μM dose of Zn^{2+} and varying doses of glutamate, a dose dependent killing effect was observed with increasing glutamate concentrations (Fig. 6.5). The 0.1 mM dose of glutamate showed no effect with Zn^{2+}, while the 1 mM glutamate/Zn^{2+} combination resulted in 23% cell death and the 10 mM glutamate/Zn^{2+} combination resulted in 33% cell death (Fig. 6.5).

Inasmuch as glutamate acts at NMDA, AMPA, and KA receptors, selective agonists were employed to investigate the mechanism of the combined Zn^{2+}/glutamate toxicity. Zn^{2+} (100 μM) combined with NMDA (500 μM) had no effect on live cell number (Fig. 6.6); however, when combined with AMPA (500 μM) or KA (500 μM), 35% cell death was observed (Fig. 6.6). CNQX, an AMPA/KA receptor antagonist, was then used to determine the extent to which Zn^{2+} could be impeded (Fig. 6.7). By itself, CNQX treatment had no effect on live cell number (Fig. 6.7A). Significant toxicity with Zn^{2+} was achieved in this experiment using a 200 μM dose of Zn^{2+} alone (30%) (Fig. 6.7A), or a 100 μM dose of Zn^{2+} in combination with 500 μM AMPA (27%) or 10 mM of glutamate (17%) (Fig. 6.7B). CNQX treatment (500 μM) given concomitantly with the combination of Zn^{2+}/AMPA or Zn^{2+}/glutamate was effective in blocking cell death (Fig. 6.7B). By contrast, CNQX treatment had no effect on the toxicity of Zn^{2+} when used alone at the 200 μM dose (Fig. 6.7A).

Finally, the neuroprotective effects of E2 were analyzed using the Zn^{2+} (100 μM) and glutamate (10 mM) combined toxicity (Fig. 6.8). The Zn^{2+}/glutamate combination resulted in a 38% decrease in live cell number, which was not affected by treatment with

GSH (3.25 μM) alone (Fig. 6.8). Suprisingly, E2 treatment alone had a significant protective effect at the 2 nM dose, and this effect was not augmented by the addition of GSH (Fig. 6.8).

6.4 Discussion

The contribution that zinc toxicity makes with regard to excitotoxic events is currently under investigation; likewise, the mechanisms behind estrogen's protection in epidemiological studies of stroke are under investigation as well. Several groups have effectively demonstrated estrogen protection from glutamate toxicity [16,71,194]; however, this is the first report of protection by estrogens with regard to zinc toxicity or zinc in combination with glutamate. The requirement of GSH for physiological relevance using zinc toxicity alone, and not for the combination, suggests that estrogens are working via more than one mechanism to achieve neuroprotection.

In stroke, there is both a core and penumbra region to consider, and the loss of neurons in these regions may be a consequence of the type of neuronal death which occurs. The type of death may be attributable to a variety of cellular insults, occurring singly or in combination, and includes excitotoxicity and/or oxidative stress. Necrosis is caused by traumatic events with a rapid collapse of internal homeostasis [24] characterized by the selective loss of membrane permeability, resulting in the swelling of organelles and subsequent rupture of the plasma membrane and is thought to occur in the core region [190]. By contrast, apoptosis is a programmed cell death characterized by cell shrinkage, membrane blebbing, and genomic fragmentation [53] and may be the type of delayed cell death seen in the penumbra region.

The ability of E2 to protect against the zinc/glutamate toxicity without GSH may indicate protection against a necrotic type cell death, in which the presence of estrogen either stabilizes the plasma membrane or blocks channels which are necessary for cell death to occur. Certainly, glutamate has been associated with excitotoxicity, and high concentrations of glutamate and other excitatory amino acids selectivley kill neurons by their depolarizing actions [152]. Other reports of glutatmate toxicity note the involvement of inhibited cystine transport as a component causing a decrease in GSH levels [144]. In the absence of excitatory amino acid stimulation, disturbances of intracellular Ca^{2+} homeostasis may also result in the alteration of cell function, cell blebbing and lysis [154]. The toxicity of Zn^{2+}, given its cationic charge, could function to disturb the Ca^{2+} balance in the presence and absence of excitatory amino acid stimulation and produce both types of cell death. If E2 protection is dependent on the presence of GSH, perhaps the type of cell death being prevented is apoptotic in nature and characteristic of the zinc toxicity alone. Certainly the other insults in which GSH plays a role in protection, like βAP and serum deprivation, are associated with apoptotic cascades [66,119] and oxidative stress may participate in apoptotic signaling [57].

Zinc is present in high concentrations in hippocampal regions known to be susceptible to ischemic events [63], including CA 1-4 radiatum and oriens, and dentate gyrus. Moreover, zinc has been localized to the synaptic boutons of excitatory neurons, especially mossy fibers. Given that excitotoxic events involve glutamate toxicity, the toxicity attributed to zinc and glutamate may be more physiologically relevant. The concentration of zinc in the hippocampus has been estimated at 210-300 μM. Further,

Assaf and Chung [6] found that depolarization released 18% of hippocampal zinc pools , and since the extracellular space has been estimated to be 15% of brain volume, it is likely that the peak extracellular concentration of zinc would be approximately 300 μM. These estimates are in line with the concentrations found to be toxic in these experiments. The contribution of glutamate to this toxicity may be substantial, as glutamate concentrations in synaptic boutons are reported to be between 60-210 mM. Taken one step further, the displacement of a competitive antagonist from NMDA receptors by glutamate showed peak concentrations of glutamate in the synaptic cleft at 1.1 mM [38], concentrations at which when combined with zinc in these experiments caused toxicity.

The function of zinc release in neurotransmission and normal brain physiology may be vastly different from the pathophysiological events that occur in ischemia/reperfusion. Zinc has a putative neuromodulatory role, with reports of potentiation of AMPA receptors and attenuation at NMDA receptors [35] and activation of γ-aminobutyric $acid_A$ ($GABA_A$) receptors [220]. Four routes of entry have been identified which allow zinc access to the cell [184]. These include voltage gated Ca^{2+}channels, NMDA receptor gated channels, Ca^{2+} permeable channels gated by AMPA or kainate, and transporter mediated exchange with Ca^{2+} under conditions favoring the reverse of the Na^{+}-Ca^{2+} exchanger [184]. The resistance of the SK-N-SH cell model to glutamate toxicity provides a unique advantage in studying zinc toxicity as the contribution of NMDA receptors may be inconsequential. The mechanism by which low concentrations of E2 provide protection against the combination of Zn^{2+} and glutamate toxicity in the absence of GSH may involve blocking L-type Ca^{2+} channels. The doses of calcium channel blockers used to provide protection

in experiments done by Freund and Reddig [64] are in line with the estrogen doses used to provide protection in these experiments. Moreover, L-type calcium channel blockers contain aromatic rings in their structures much like estrogen and have side effects consistent with estrogenic properties.

Another consideration with regard to zinc as a metal, in both normal and pathophysiology, is the ability of E2 and GSH to chelate or otherwise interfere with its function [123]. Zinc is redox inert, and cannot utilize changes in oxidation states to mobilize or change compartments. Zinc excretion is relatively uninfluenced by changes in diet or by zinc infusion, but can be affected by HRT in some women [91]. Whether E2 can chelate Zn^{2+} is unknown. The effects of GSH and GSSG on Zn^{2+}status are more complex. The thiol-disulfide redox state, determined by the GSH/GSSG couple, is one component in the cellular redox balance. Since both the GSH/GSSG couple and the thiolate sulfur from cysteine side chains in proteins are metal ligands and can bind Zn^{2+}, it is possible that changes in thiol–disulfide balance will affect metal binding and vice versa [123]. GSSG can interact with metallothioneins and release Zn^{2+} ions in a monophasic fashion [123]. Indeed, further studies are needed to establish conditions under which the availability of Zn^{2+} is controlled by interactions between metallothionein and GSH [123].

Finally, if the above is true, conditions in which redox stress is a component of disease could lead to the abnormal release of intracellular Zn^{2+} from proteins, and contribute to the cascade of pathophysiological events linking diseases like AD and stroke. In AD, Zn^{2+} has been shown to block amyloid protein calcium channels [4] and induce amyloid aggregation [27] and both zinc and glutathione have been implicated in the

biological aging process. Perhaps there is an important link between GSH and Zn in the cellular redox balance which is offset by estrogens. The rest of this dissertation investigates the contribution of GSH content and the activity of glutathione peroxidase and glutathione reductase which could contribute to the observed neuroprotection seen with estrogens.

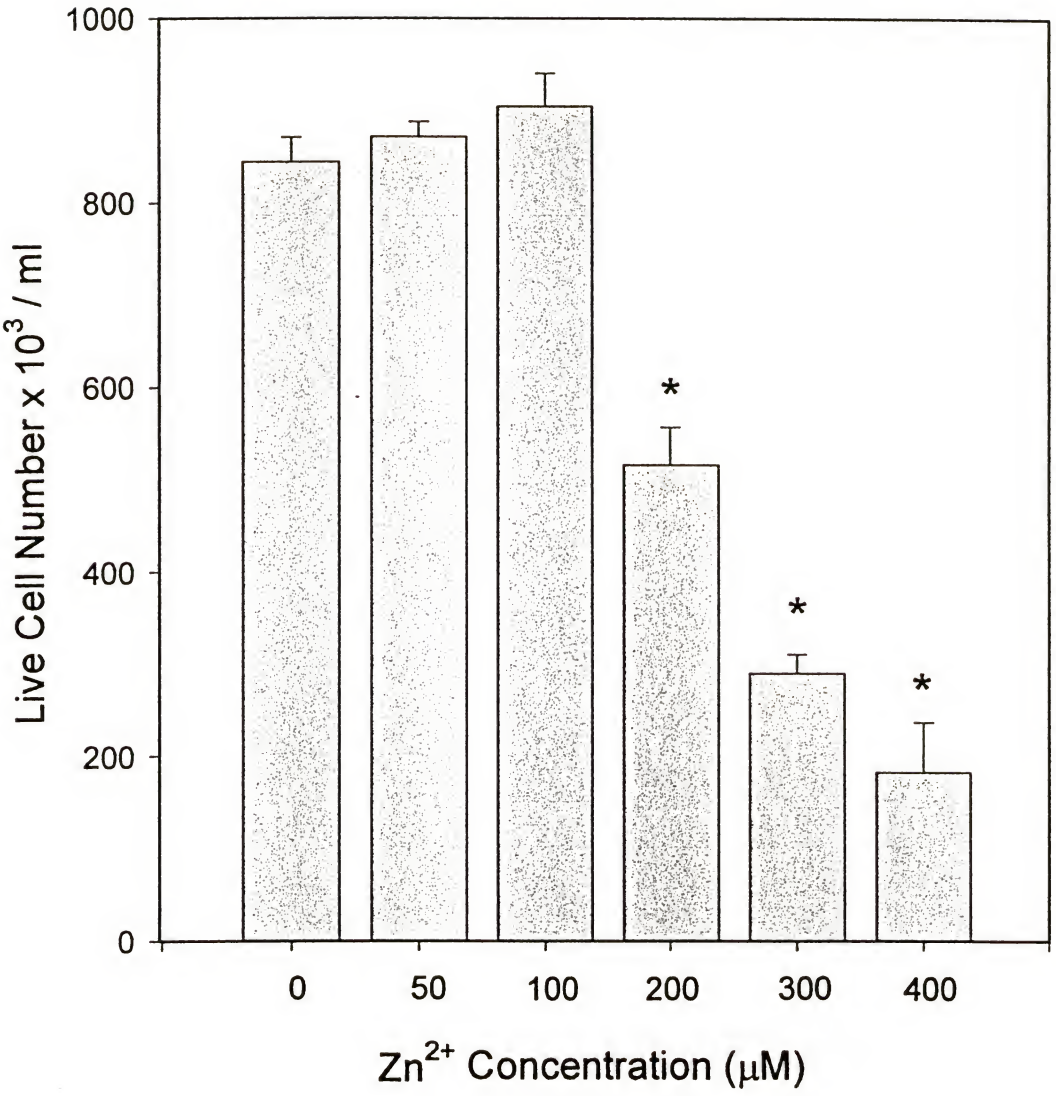

Fig. 6.1. Dose response of Zn^{2+} on SK-N-SH neuroblastoma cells. Cells plated at 10^6 cells/well were exposed to Zn^{2+} for 20 h of treatment. The number of trypan blue excluding cells is depicted as live cell number using mean values ± SEM for n = 4 wells per group. LD_{50} = 248 ± 24.1 μM. *$p<0.05$ when compared to the 0 dose group as determined by ANOVA followed by Tukey's test post hoc.

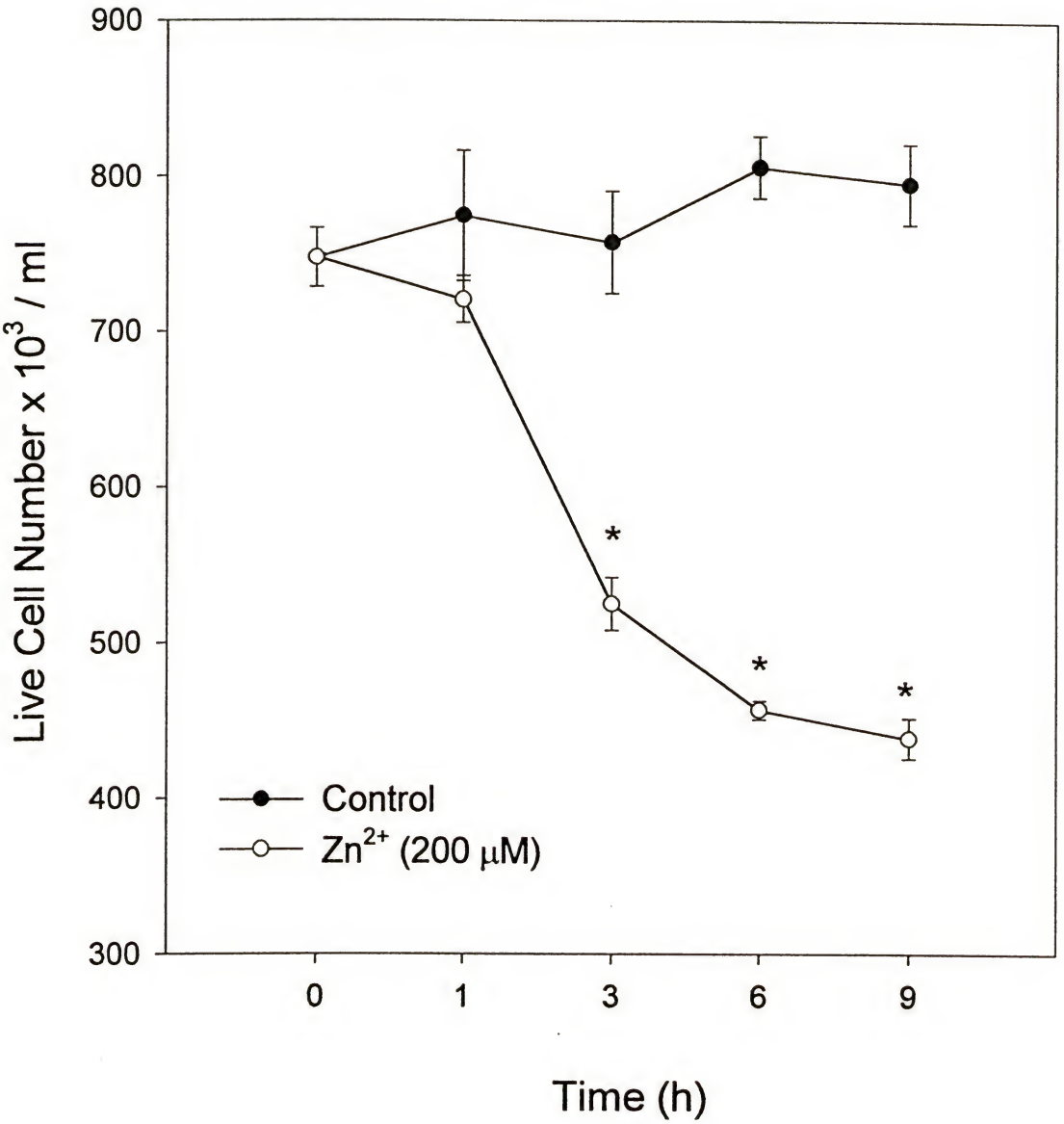

Fig. 6.2. Time course of Zn^{2+} effects on live cell number. Cells plated at 10^6 cells/well were exposed to Zn^{2+} (200 μM) for the indicated times. The number of trypan blue excluding cells is depicted as live cell number using mean values ± SEM for n = 4 wells per group. *$p<0.05$ when compared to controls as determined by ANOVA followed by Tukey's test post hoc.

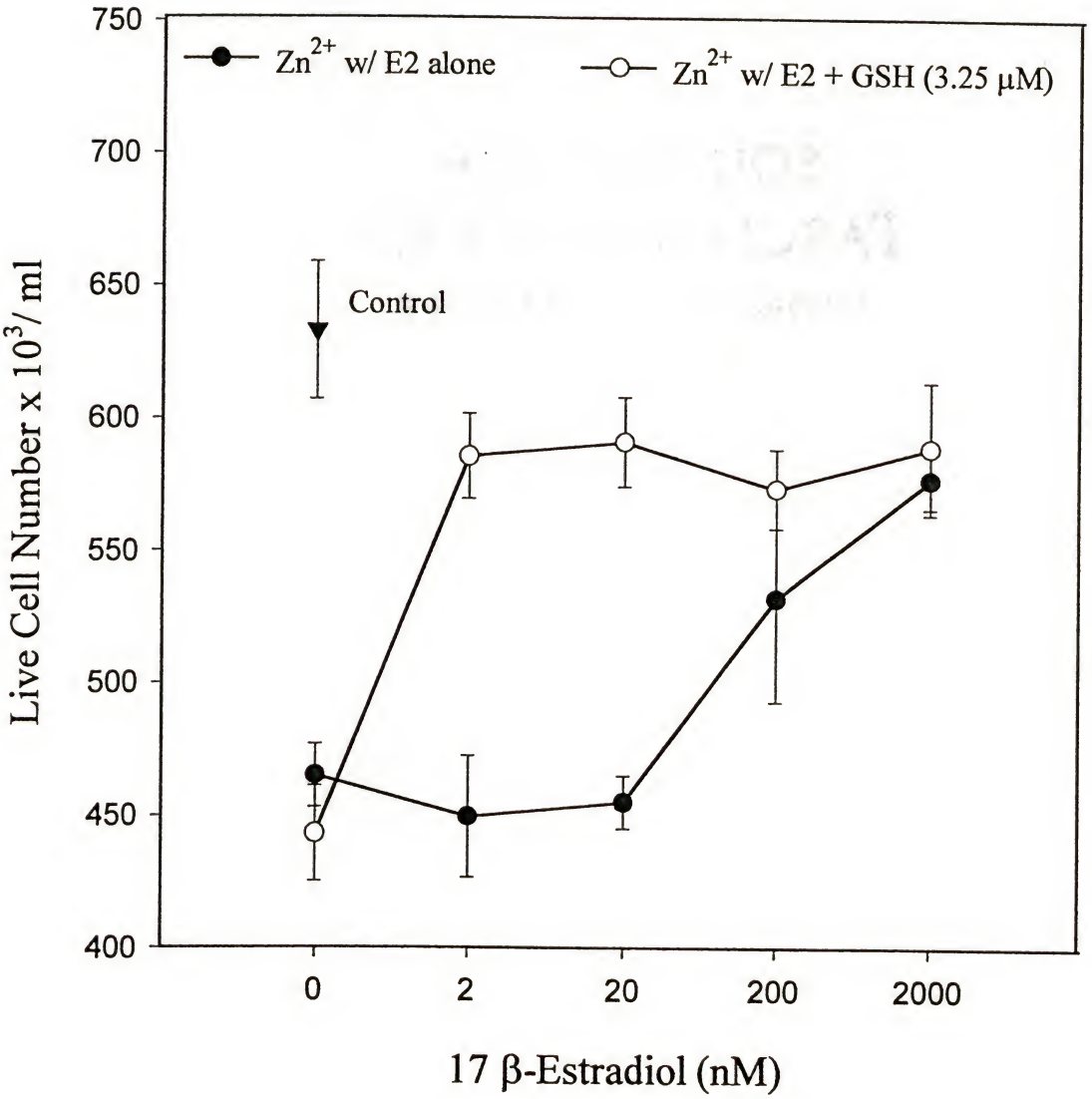

Fig. 6.3. Dose response effects of 17 β-estradiol (E2) on Zn^{2+} toxicity in the presence and absence of GSH. Cells plated at 10^6 cells/well were subjected to Zn^{2+} (200 μM) for 20 h of treatment in the presence and absence of increasing doses of E2 with or without GSH (3.25 μM). Depicted are mean values ± SEM for n= 3-4 wells per group. Two way ANOVA revealed significant effects of dose of E2 and presence of GSH (F=4.774; p=0.002) in the protection against Zn^{2+} toxicity.

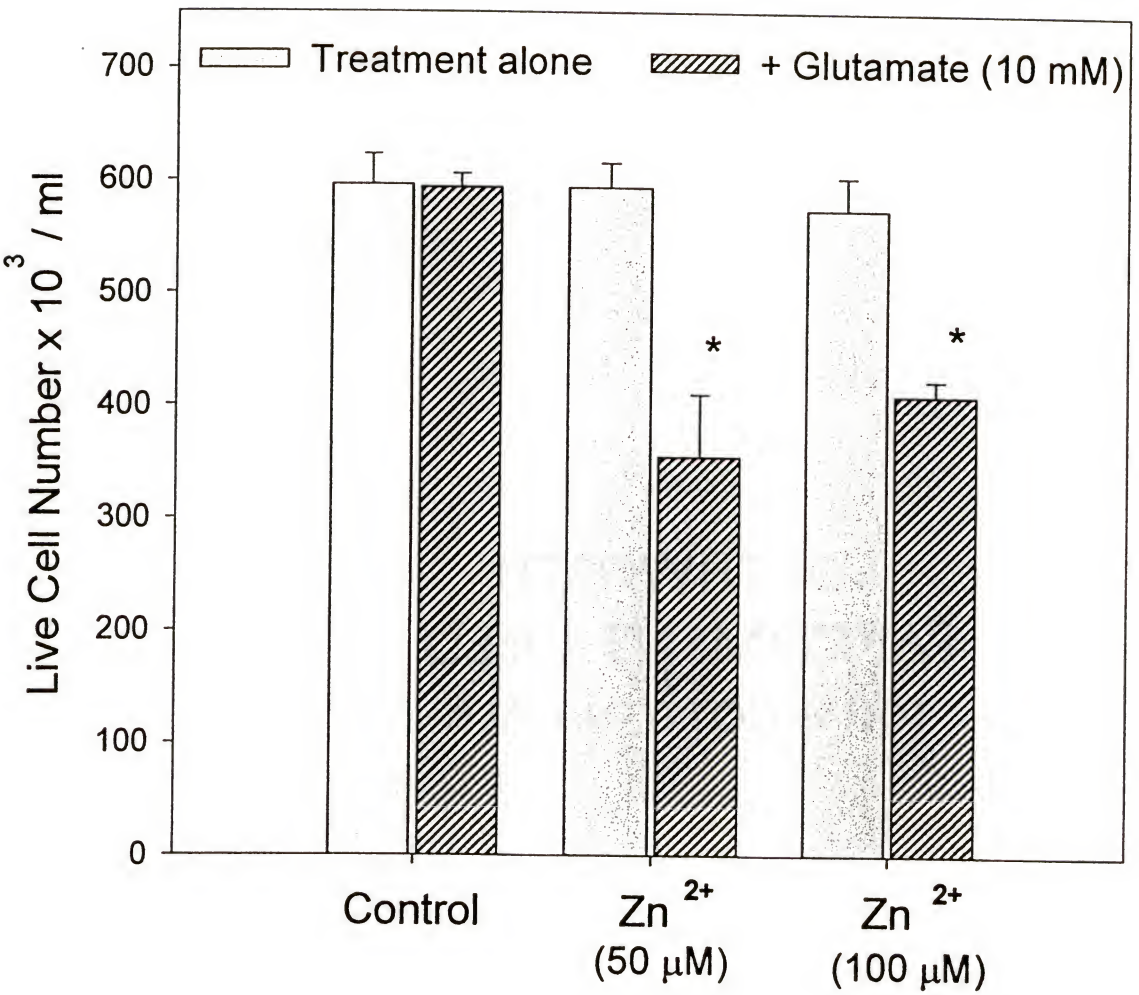

Fig. 6.4. Effects of glutamate addition to non-toxic doses of Zn^{2+} in SK-N-SH cells. Cells plated at 10^6 cells/well were exposed to Zn^{2+} in combination with glutamate for 20 h. Live cell number determined by trypan blue exclusion. Depicted are mean values ± SEM for n = 4 wells per group. *$p < 0.05$ when compared to the respective Zn^{2+} dose group as determined by ANOVA followed by Tukey's test post hoc.

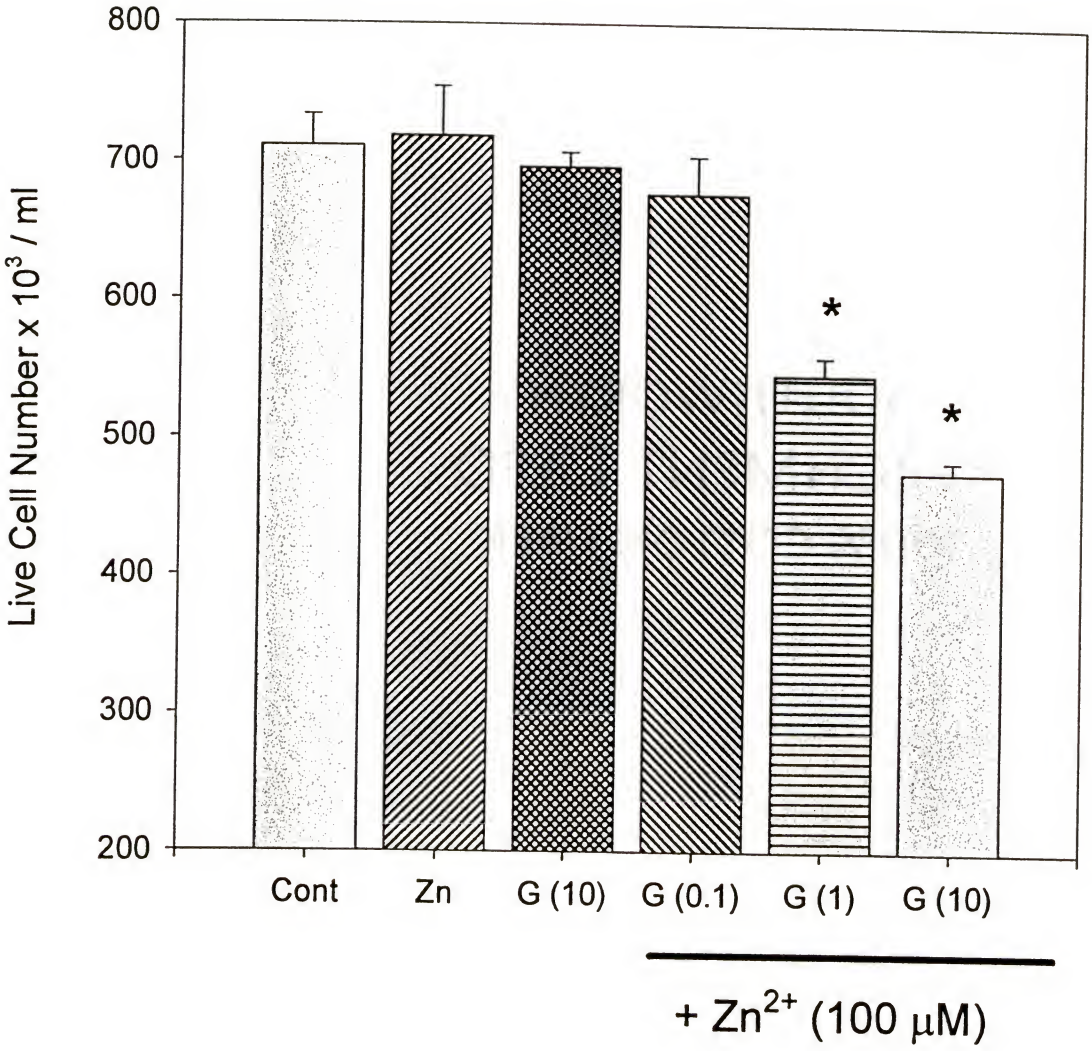

Fig. 6.5. Effects of Zn^{2+} addition to non-toxic doses of glutamate in SK-N-SH cells. Cells plated at 10^6 cells/wee were exposed to glutamate (10 mM) or glutamate (0.1-10 mM) in the presence of a non-toxic dose of Zn^{2+} (100 μM) for 20 h. Live cell number determined by trypan blue exclusion. Depicted are mean values ± SEM for n = 4 wells per group. *$p<0.05$ when compared tocontrols as determined by ANOVA followed by Tukey's test post hoc.

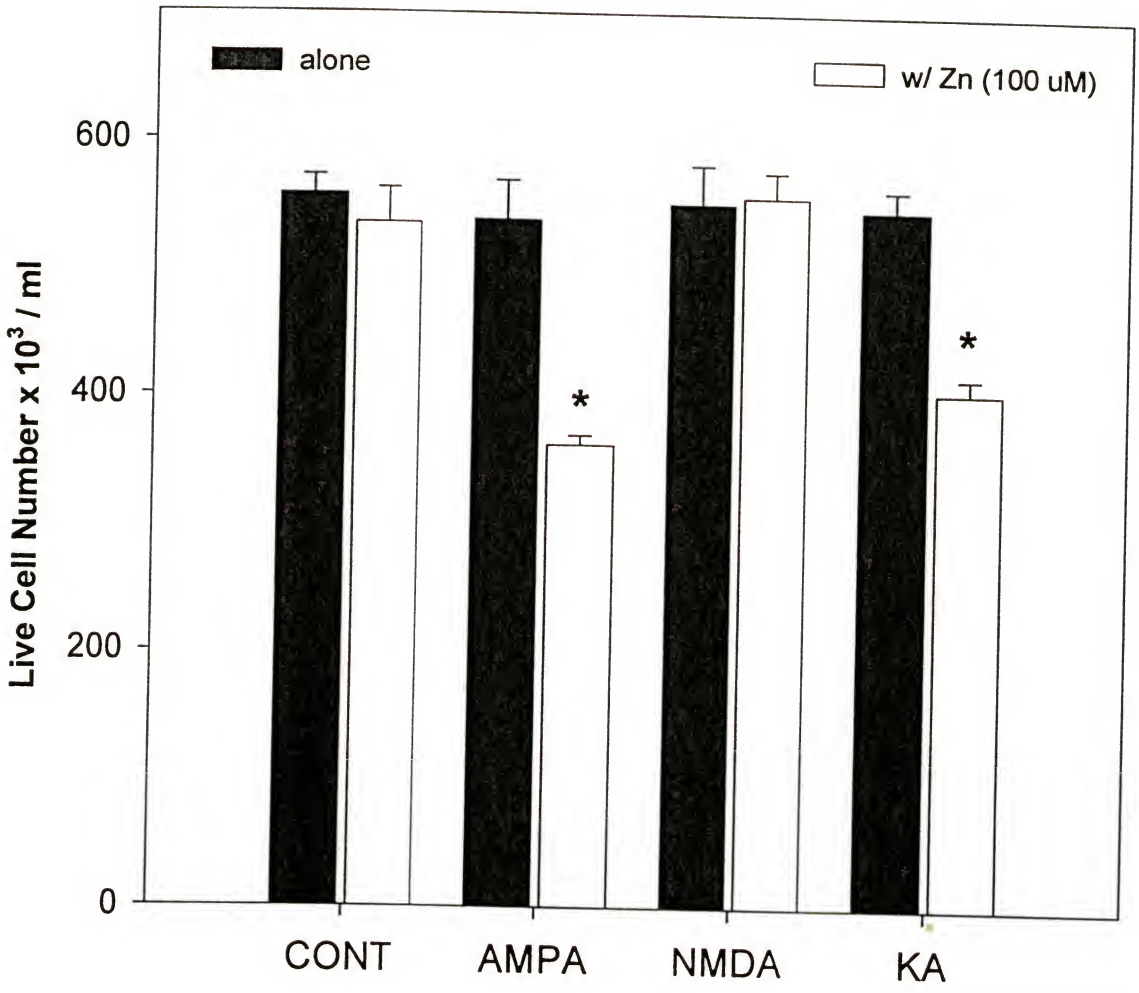

Fig. 6.6 AMPA, NMDA, or kainic acid (KA) in 500 μM doses were combined with Zn^{2+} to evaluate the receptors involved in the toxicity associated with the Zn^{2+} /glutamate combination. Depicted are mean values ± SEM for n = 4 wells per group. *$p<0.05$ as determined by ANOVA followed by Tukey's test post hoc.

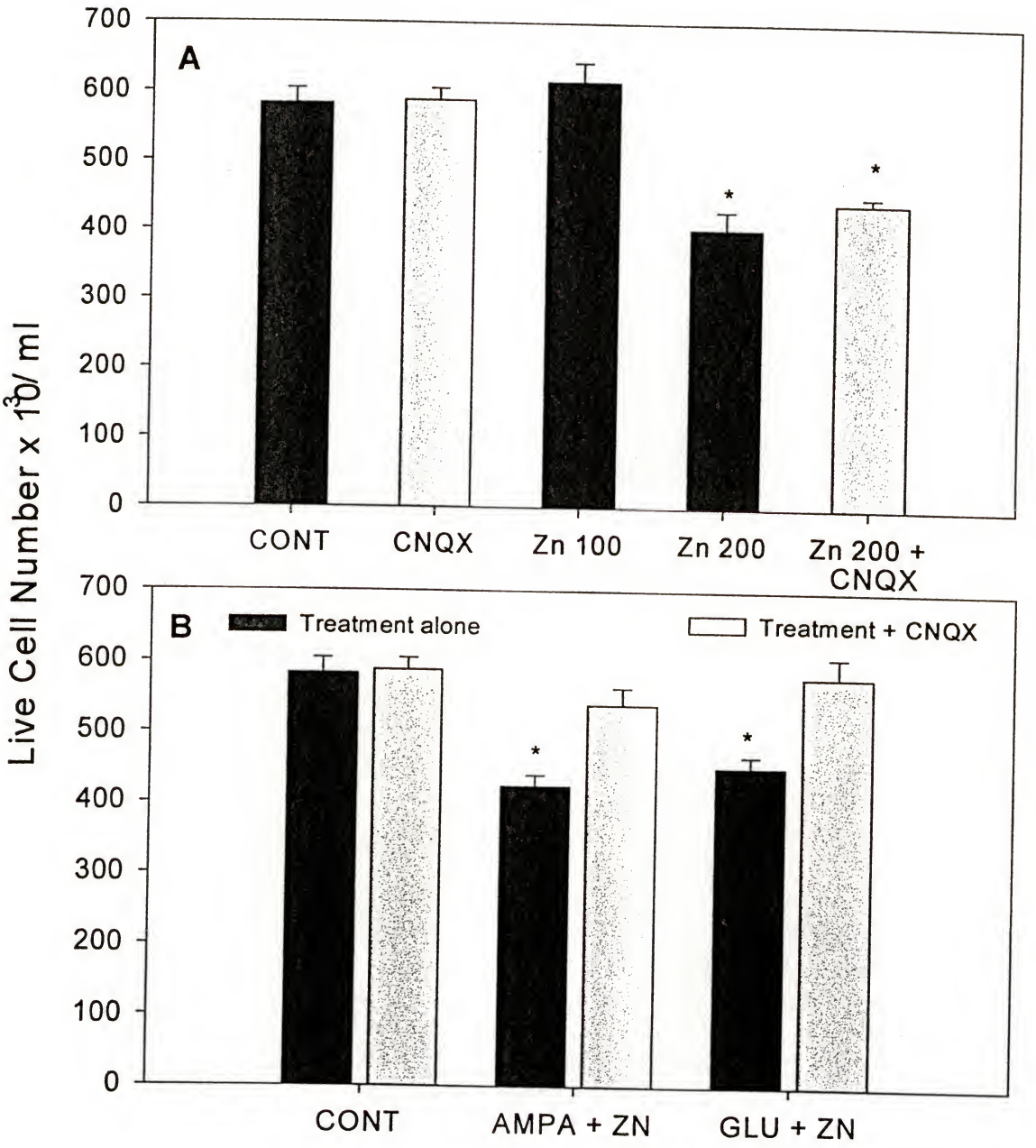

Fig 6.7. Effects of the AMPA antagonist CNQX on Zn^{2+} toxicity alone or in combination with glutamate or AMPA. A) SK-N-SH cells subjected to Zn^{2+} alone (200 μM) and CNQX, and B) Zn^{2+} in combination with glutamate (10 mM) or AMPA (500 μM) in the presence or absence of CNQX (500 μM). Represented are mean live cell numbers ± SEM for n = 4 wells per group. *$p<0.05$ as determined by ANOVA followed by Tukey's test post hoc.

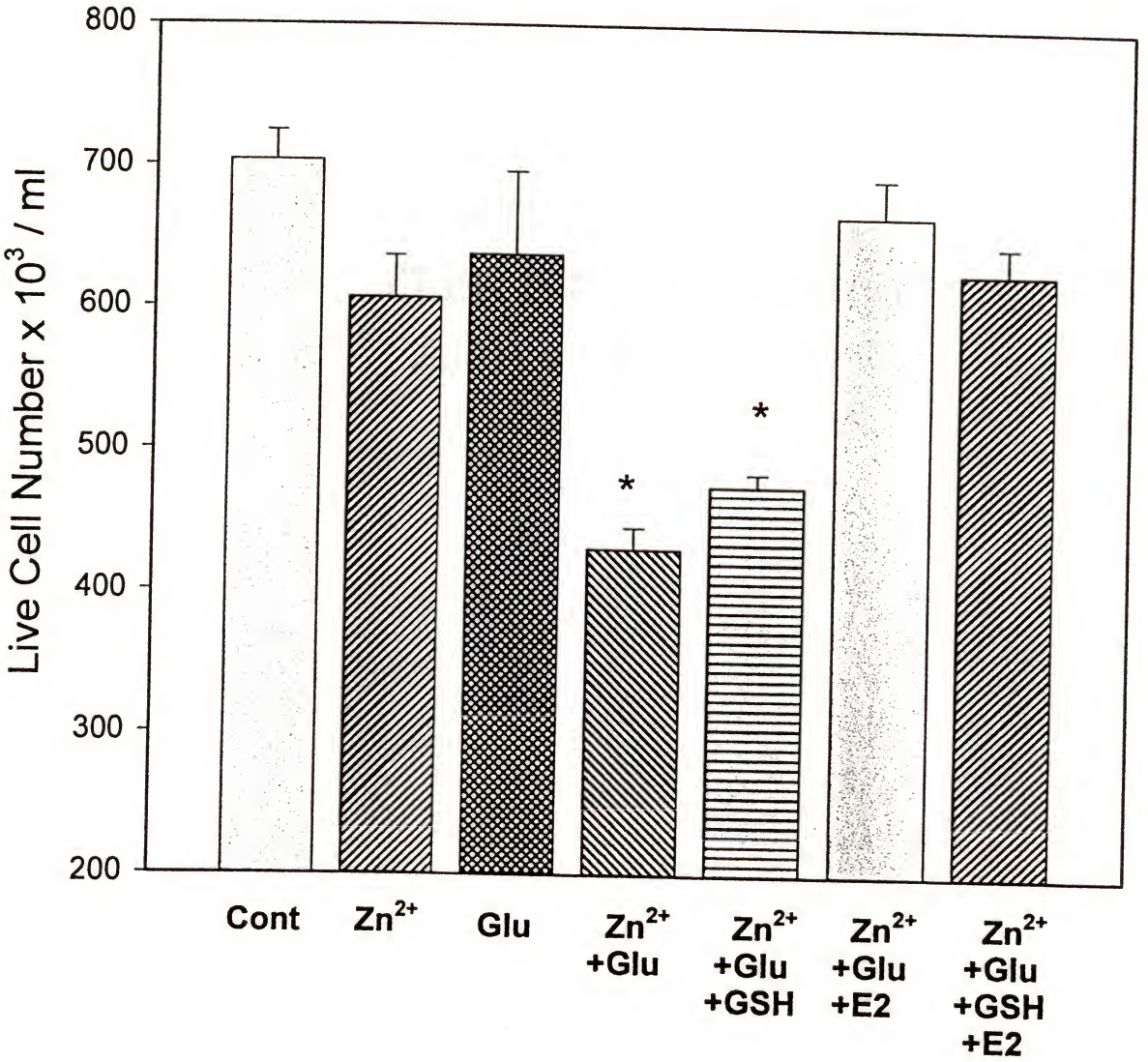

Fig. 6.8. Neuroprotection demonstrated by E2 (2 nM) using toxicity attributed to the combination of Zn^{2+} and glutamate. SK-N-SH cells were exposed concomitantly to 20 h or Zn^{2+} (100 μM) with glutamate (10 mM) in the absence or presence of E2 (2 nM), GSH (3.25 μM), or the combination. The number of trypan blue excluding cells is depicted for n=4 wells per group and shown as mean value ± SEM. *$p<0.05$ when compared to controls as determined by ANOVA followed by Tukey's test post hoc.

CHAPTER 7
EVALUATING THE CONTRIBUTION OF INTRACELLULAR GSH CONTENT TO THE NEUROPROTECTION PROVIDED BY THE COMBINATION OF ESTROGEN AND GLUTATHIONE

7.1 Introduction

Increasing the amount of GSH available for participation in antioxidant activities required as a result of βAP treatment or zinc toxicity may be one mechanism of protection provided for by the E2-GSH interaction. The intracellular level of GSH in mammalian cells is 0.5-10 mM, whereas micromolar concentrations are typically found in blood plasma and extracellular environments [133]. Given that E2 levels are in the low nanomolar range and GSH is present in much higher amounts than E2, it is possible that the E2-GSH interaction participates in the neuroprotection of cells by increasing the intracellular pool of GSH or by preventing cellular GSH depletion.

The structure of GSH was elucidated following observations of its action [45,46], its degradation [88] and its synthesis [49]. Glutathione is a tripeptide made up of glutamate, cysteine and glycine and is called γ-glutamylcysteinylglycine because the glutamate-cysteine peptide linkage is at the terminal or γ carboxyl group of glutamate. Of particular interest is the thiol group on the cysteine moiety which is capable of undergoing one- and two- electron redox reactions, allowing GSH to be oxidized to its dimer GSSG and subsequently reduced back to its constituent molecules of GSH. GSH can form free

radicals; however, the GSH free radical is relatively unreactive and often only terminates on contact with another GSH or with other proteins containing thiols groups [41].

The maintenance of intracellular levels of GSH is a dynamic process, integrating functions in metabolism, transport, and antioxidant protection. De novo synthesis occurs in two separate enzymatic reactions, characterized by Bloch and colleagues [199,200] each requiring one molecule of ATP. The first step involves the formation of the γ-glutamyl linkage described above, and is catalyzed by γ-glutamyl cysteine synthetase (gGCS). The enzyme glutathione synthetase catalyzes the second reaction, resulting in addition of glycine to the γ-glutamyl-cysteine molecule and thus forms GSH in the reduced state.

The biosynthesis of GSH is regulated by feedback inhibition, whereby GSH inhibits formation of γ-glutamyl cysteine [171] and ADP has been reported to be a competitive inhibitor of glutathione synthetase [200,221] with the exception of the human red blood cell enzyme [95]. Lastly, de novo synthesis appears to be strongly limited by the availability of substrates, with the uptake of cysteine as the rate limiting amino acid [207].

Synthesis occurs primarily in the cytoplasm of the cell although detoxification, antioxidant, and physiological reactions occur in other cellular compartments [132]. GSH is supplied to the mitochondria through an energy-dependent transporter that is coupled to anion efflux and the mitochondrial GSH pool makes up about 20-30% of the total cellular GSH content [196]. The persistent nuclear GSH pool makes up approximately 1-2% of the total intracellular glutathione and GSH in this compartment participates to prevent

oxidation of critical protein sulfhydryls which function to maintain the efficiency of DNA repair enzymes [196].

The synthesis and compartmentation of GSH is balanced by the conjugation of GSH to other molecules by glutathione S-transferases, and the use of GSH as a substrate for the glutathione peroxidase reaction described previously. The GSSG dimer formed from this reaction can be reduced using the enzyme glutathione reductase. Efflux can occur from the cell in the forms of GSH, GSSG, and GS- in conjugation with other proteins. Catabolism of GSH occurs by the enzyme glutathione transpeptidase located on the extracellular membrane [80] and is thought to be unaffected by other peptidases due to its γ peptide linkage.

An evaluation of the activity of GSH as an antioxidant must consider the concentration of GSH and the redox state of the GSH/GSSG pool [178]. Mechanisms of neuroprotection which utilize GSH as a substrate may be dependent solely on the concentration of GSH while the redox state of GSH may regulate cell signaling processes. The purpose of this chapter is to examine the intracellular content of GSH, to manipulate the intracellular GSH content to determine effects on viability, and to determine the redox state of cells subjected to βAP in the presence and absence of E2, GSH, and the combination, to gain further insight into the E2-GSH interaction.

7.2 Materials and Methods

Materials Lyophilized βAP and E2 solutions were made as previously described. DL-buthionine-S,R-sulfoximine (BSO), glutathione monoester (MONO), n-acetyl-L-cysteine (NAC), and glutathione disulfide (GSSG) were initially dissolved in cell culture

media and diluted to the concentrations indicated. Materials used for the Tietze assay, 5,5 dithiobis-(2-nitrobenzoic acid) (DTNB), NADPH, and the glutathione reductase enzyme (GRD) were made up in buffer as dictated [209]. Sulfasalicylic acid (SSA; 1% w/v), and undiluted 2-vinyl pyridine (Aldrich) are used as indicated in chapter 3. Unless otherwise noted, materials were obtained from Sigma Chemical Corp.

SK-N-SH Neuroblastoma Cell Culture SK-N-SH cells used in the following experiments were in passes 32-58. Studies for cell viability were initiated by plating 1 x 10^6 cells per well in 24 well plates, allowing attachment in regular media and then decanting that media and replacing with the appropriate treatment after 4 h. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Grand Island, NY), supplemented with 10% charcoal stripped FBS with absolute ethanol (34 μM) as a vehicle control, or the addition of βAP 25-35 (20 μM), E2 (2 - 200 nM), GSH (3.25 μM), MONO (3.25 μM), NAC (100 μM) , GSSG (1.6 μM), BSO (0-500 μM) or a combination as indicated. SK-N-SH cell viability was determined utilizing the trypan blue exclusion method after 24 h of incubation.

GSH/GSSG content SK-N-SH neuroblastoma cells were plated at 4 x 10^6 cells/plate and incubated in 100 mm dishes until 80% confluency was obtained. At this time, media was decanted and treatments added as appropriate. Following treatment for 24 h, cells were incubated in 0.9 ml Versene for lifting, triturated to ensure even pipetting, and subsequently divided into three equal aliquots, one each for cell viability determined by trypan blue exclusion, total glutathione content and GSSG assay as described previously in Chapter 3.

Reduced GSH assay Reduced GSH was analyzed using a kit obtained from Calbiochem (La Jolla, CA). Cells incubated for 24 hours as previously described were lifted in EDTA (200 µl) and put into microcentrifuge tubes. The cells were spun at 1500 rpm and the EDTA removed from the cell pellet, and the cells were resuspended in 100 µl metaphosphoric acid (5% w/v) to precipitate proteins and sonicated to break open cell membranes. The cells suspensions were then centrifuged at 3000 rpm for 15 minutes at 4° C. One hundred µl aliquots of the supernatant are used for GSH assay according to manufacturer's instructions. To the supernatant, 80 µl of buffer and 10 µl of both the R1 and R2 reagents were added to give a final volume of 200 µl in a microtiter tray, which was incubated at room temperature for at least 10 minutes in the dark and read on the SLT® tray reader at 400 nm.

7.3 Results

Total GSH content obtained by the Tietze method measures the GSH, GSSG, and GS- moieties conjugated to other thiols, while the results obtained using the kit from Calbiochem strictly measures the GSH content of cells. Similar results were obtained for each method employed (Fig. 7.1) with the Tietze method (680 ± 171 ng/10^6 live cells), which includes GSSG and GS- moieties (6.18 ng/10^6 total cells) as compared to the Calbiochem method (752 ± 428 ng/10^6 total cells). When the GSSG content is subtracted from the values obtained from the Tietze method according to the equation (Total-2*GSSG =GSH), the result is 667.6 ng/10^6 total cells. The Tietze method was employed for the remainder of the studies as the Calbiochem method was more variable and limited to the measurement of GSH which precluded studies of redox potential.

The total GSH content of cells for controls cells or cells treated with E2 (2 nM), GSH (3.25 μM), or the combination, all in the absence of toxicity, was not statistically different from each other using one way ANOVA, (Fig. 7.2) and the pooled average is 564 ± 107 ng/10^6 total cells. Likewise, the GSH content of these same groups subjected to βAP toxicity were not determined statistically different (Fig. 7.2), and the pool average was 275±102 ng/ 10^6 total cells. All of the βAP treated groups were different from the controls, and statistics on pooled data show significant effects of βAP treatment ($p<0.001$) when subjected to a t-test followed by Mann-Whitney Rank sum. The GSSG content of cells, determined by derivatization of all reduced GSH using 2-vinyl pyridine, was examined for these same treatments (Fig. 7.3) with no significant difference noted between groups. Pooled data shows mean values of 6.13 ± 3.22 ng/10^6 total cells for all controls, while the βAP treated groups averaged 5.58 ± 5.54 ng/10^6 total cells. It is important to note that the GSH in these samples was well below the concentration at which GSH contamination might have an affect on these results.

The ratio of GSH:GSSG was evaluated for the means of the pooled controls versus pooled βAP treated cells using the above equation. This ratio was 44:1 in the reduced pools after βAP treatment of cells, versus 92:1 for the various treated control cells. This measure has been used quite often as a determination of oxidative stress, while others employ the Nernst equation to evaluate the redox potential of the GSH-GSSG exchange. Since these measures are calculated on the basis of GSH and GSSG concentrations, significantly different results should be re-evaluated using these criteria. In the case of the pooled controls and βAP treated cells, the electrochemical potentials are

calculated according to the equation $E_h = E_0 + RT/2F \ln [GSSG]/[GSH]^2$, using -240 mV as E_0 [173] which is an estimate of the standard potential for the 2 GSH/GSSG couple, and using data given in Fig. 2 and Fig. 3. The results are -381 mV for the control treated cells and -361 mV for βAP treated cells.

Manipulation of the intracellular GSH concentration was attempted using compounds demonstrated to increase GSH content, such as n-acetyl cysteine (NAC) and glutathione monoester (MONO) (Fig. 7.4). The GSH content proved unaffected by the addition of GSH (3.25 μM) as compared to controls (Fig. 7.4a), while the addition of NAC increased significantly increased GSH content of cells by 41%, and MONO treatment increased GSH content by 22% when compared to controls (Fig. 7.4a). It should also be noted in this discussion that GSSG treatment increased GSH content by 22%. The GSSG content of these cells was also measured (Fig. 7.4b). No significant difference was obtained with the NAC, GSH or GSSG groups; however, MONO treatment significantly increased GSSG concentration by 1.43-fold when compared to controls. In cells pre-treated with NAC, which increases GSH content, and MONO which increases GSSG content, for 24 h and then subjected to βAP treatment and increasing doses of estrogen for an additional 24 h, no effect was seen on cell viability when compared to cells that were not pre-treated (Fig 7.5). Lastly, co-treatment with either NAC or MONO in the presence or absence of E2 (2 nM) for 24 h (Fig. 7.6) showed no effect on cell viability as measured by trypan blue exclusion, while co-treatment with GSH (3.25 μM) showed protective effects consistent with those seen previously in the presence of E2.

By contrast, treatment with BSO, a compound known to deplete intracellular GSH concentrations, negatively impacts cells when subjected to βAP toxicity in the absence of E2 and GSH. The 100 μM concentration of BSO, determined to be non-toxic to SK-N-SH cells (Fig. 7.7a) was evaluated for effects on GSH and GSSG content (Fig. 7.7b and 7.7c). Twenty-four hours of BSO treatment significantly depleted the total GSH content of the cells from 826±45.5 to 83.2±11.3 ng/10^6 total cells, approximately 90% of the glutathione pool as compared to control treated cells (Fig. 7.7b), with the GSSG content similarly affected (Fig. 7.7c). When cells were pre-treated with BSO (100 μM) for 24 h and subjected to βAP toxicity in the presence of increasing doses of E2 for an additional 24 h, a significant effect was found on the viability of cells with BSO treatment using two way analysis of variance ($p<0.001$; $F=32.162$) (Fig. 7.8). For both BSO pre-treated cells and non-pre-treated cells, the 200 nM dose of E2 was effective in saving cells from βAP toxicity, which is in agreement with earlier studies. Interestingly, in cells treated with βAP and co-treated with BSO (100 μM), E2 (2 nM), GSH (3.25 μM), or a combination as indicated (Fig. 7.9), no effect of BSO on the save in live cell number elicited by the E2-GSH combination was identified using three way analysis of variance.

7.4 Discussion

The GSH pool of SK-N-SH cells is depleted in response to βAP toxicity; however, the protection afforded by the E2-GSH interaction does not appear to be correlated with cytoplasmic GSH status. Three lines of evidence support this idea: 1) SK-N-SH cells are still protected by the E2-GSH combination in the presence of βAP and a well-known GSH depleting agent; 2) compounds which increase the intracellular GSH have no

neuroprotective effects when given under pre-treatment or co-treatment conditions with E2; and 3) the GSH content of cells is unaffected by E2-GSH treatment in the presence or absence of toxicity.

Inasmuch as the concentration of GSH is 92 times more prevalent in control cells than GSSG and the concentration of GSSG remains unchanged in the presence of toxicity, the depletion of GSH must involve export from the cell. Intracellular content has been reported to be 99% GSH [133] and GSH is the major transport form. In erythrocytes, GSSG transport has been reported [109] and one could speculate that perhaps under conditions where oxidative stress is experienced by the cell, GSSG transport occurs.

The effects of BSO on the cellular GSH pool have been characterized by other groups and the time frame and doses for depletion are similar [174,214]. The mechanism for BSO depletion of GSH levels is the non-competitive inhibition of the γGCS enzyme in the first step of de novo synthesis [82]. It is important to note that BSO depletes the cytoplasmic pool of GSH, while the mitochondrial [83] and nuclear pools [51,101] of GSH remain unaffected. Given this information, it may be plausible that the E2-GSH interaction is exerting its protective effects in the mitochondria, the nucleus or the extracellular space.

The lack of protection offered by the agents which increase intracellular GSH suggests that the GSH pool of the cells is not interacting with E2 for neuroprotection. Perhaps more interesting is the lack of protection offered by the co-treatment of the GSH monoester compound. E2 structural requirements for neuroprotection have been well defined in a serum deprivation model and include the presence of an intact hydroxyl group

[15,76] and at least three rings of the steroid molecule [76]. It may be that the E2-GSH interaction depends on a structural requirement for GSH which is blocked by the addition of the monoester.

Lastly, the intracellular content of GSH in SK-N-SH cells appears to be unaffected by the E2-GSH combination in the presence or absence of toxicity, which supports the concept that the GSH depletion of cells in response to βAP toxicity might be a secondary event. The current dogma would dictate that conditions which increase oxidative stress would utilize GSH reducing equivalents for antioxidant defenses and βAP toxicity has been associated with oxidative stress as discussed in chapter 2. The observation that E2-GSH treatment saves cells but leaves intracellular GSH unaffected suggests other mechanisms for the protective interaction. There are reports of micromolar concentrations of estrogen added to IMR 32 neuroblastoma cells with the demonstration of slightly increased (non-significant) GSH concentrations; however, these levels of E2 are not physiologically relevant [22]. One important aspect of these observations is that the IMR 32 neuroblastoma cells show similar GSH values (0.99±0.09 μg/ 10^6 cells) [22] to SK-N-SH cells, offering validation for the measurements above. Gene expression is sensitive to redox state [1], and changes in redox status affect apoptosis [122]. The implication of the BSO data is that one cannot rule out the mitochondria or the nucleus as important areas for consideration; yet it highlights the idea of an extracellular mechanism between E2 and GSH for neuroprotection.

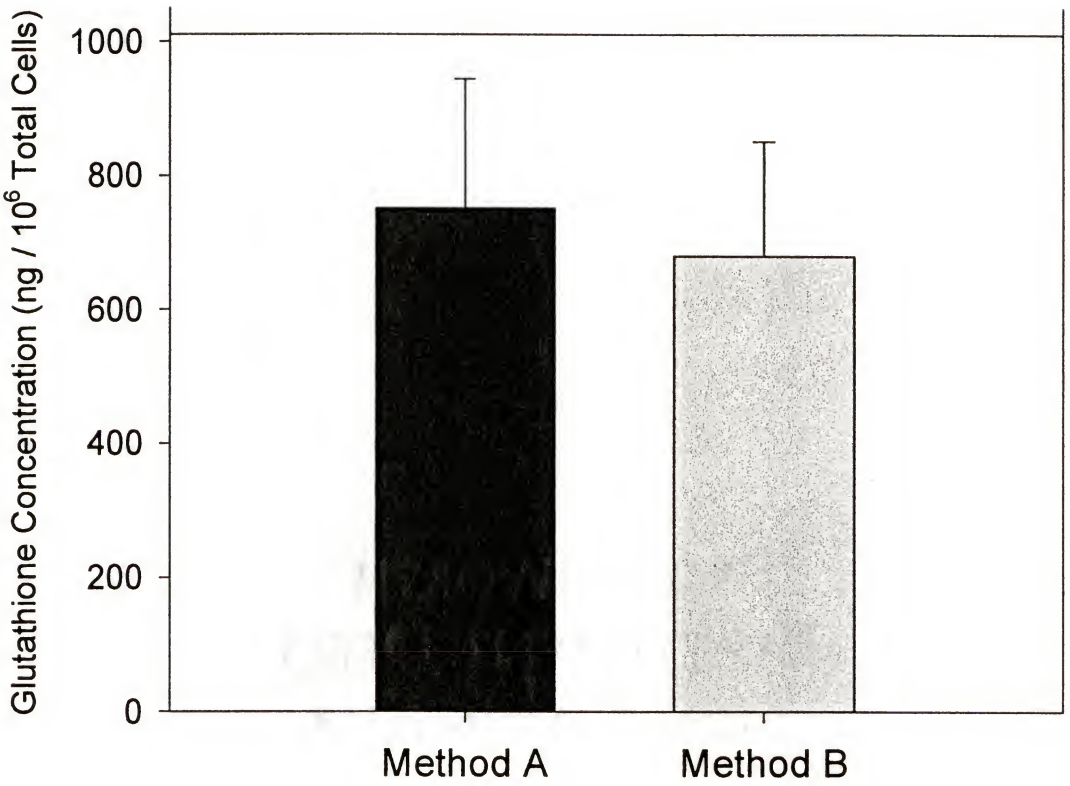

Fig. 7.1. Glutathione concentrations as obtained by two methods. Method A is a kit obtained from Calbiochem, and n = 4 wells per group, Method B is the Tietze method, for n = 3-4 wells per group with 4 replications. Depicted are mean values ± SEM.

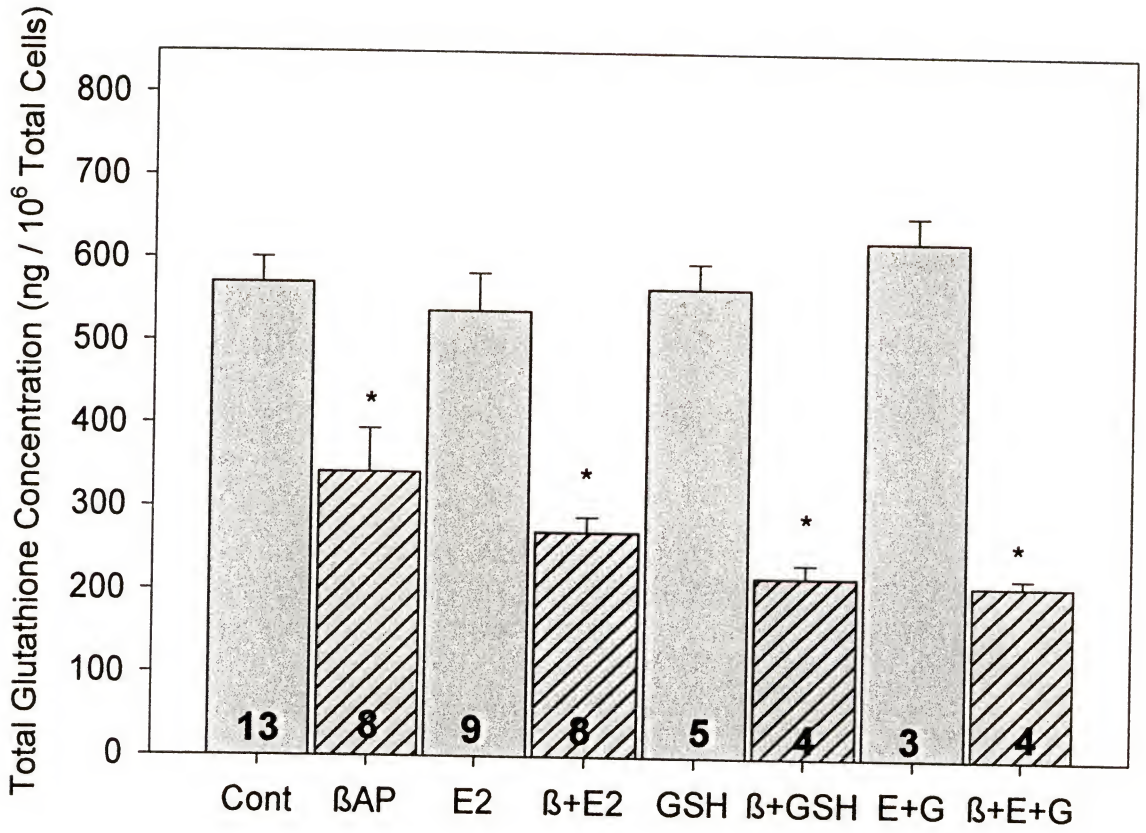

Fig. 7.2. Effects of treatment with E2, GSH, βAP or the combination on the total glutathione content of SK-N-SH cells. Cells plated in 100 mm dishes were allowed to grow to 80% confluency and treated as indicated for 24 hours. Depicted are mean values ± SEM for the number of dishes indicated on each bar. *$p<0.05$ versus controls as determined by ANOVA followed by Tukey's test post hoc.

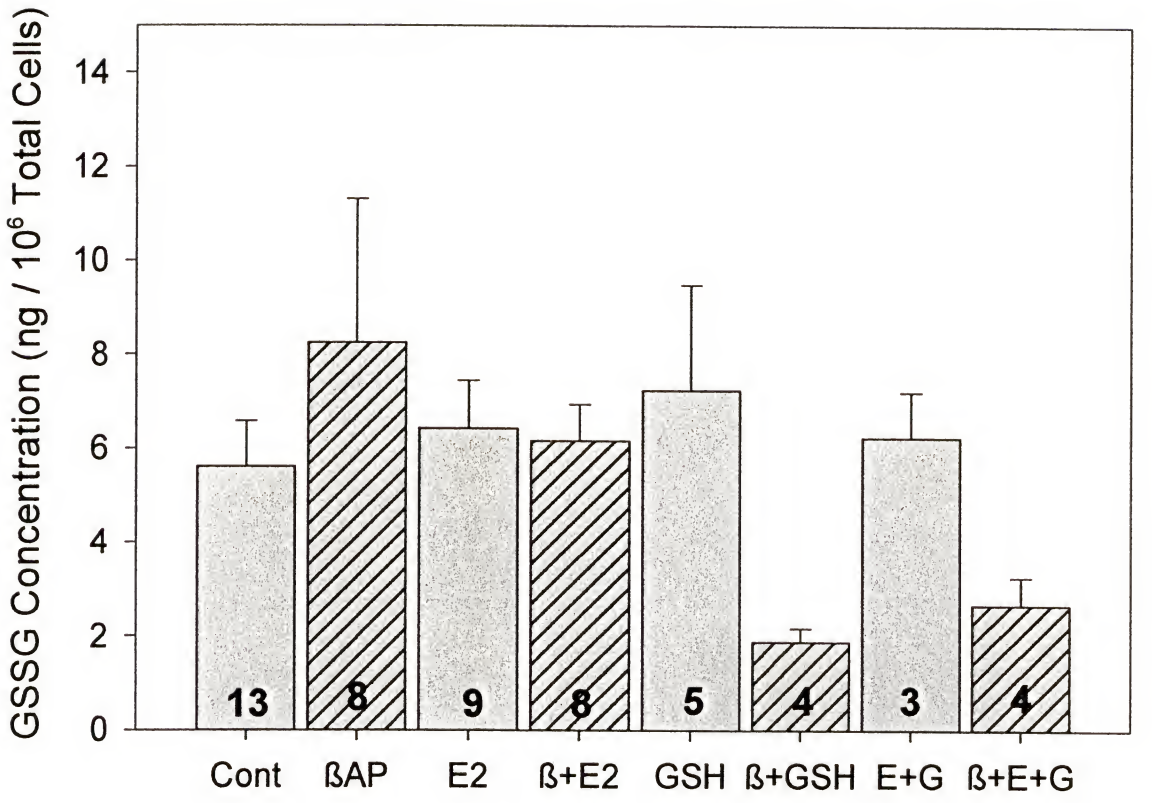

Fig. 7.3. GSSG concentration in SK-N-SH cells after treatment with E2 (2 nM), GSH (3.25 μM), βAP (20 μM), or the combination as indicated for 24 h. Cells plated in 100 mm dishes were grown to 80% confluency and assayed as stated in Chapter 3. Depicted are mean values ± SEM for the number of dishes indicated on each of the bars. *$p<0.05$ versus controls as determined by ANOVA followed by Tukey's test post hoc.

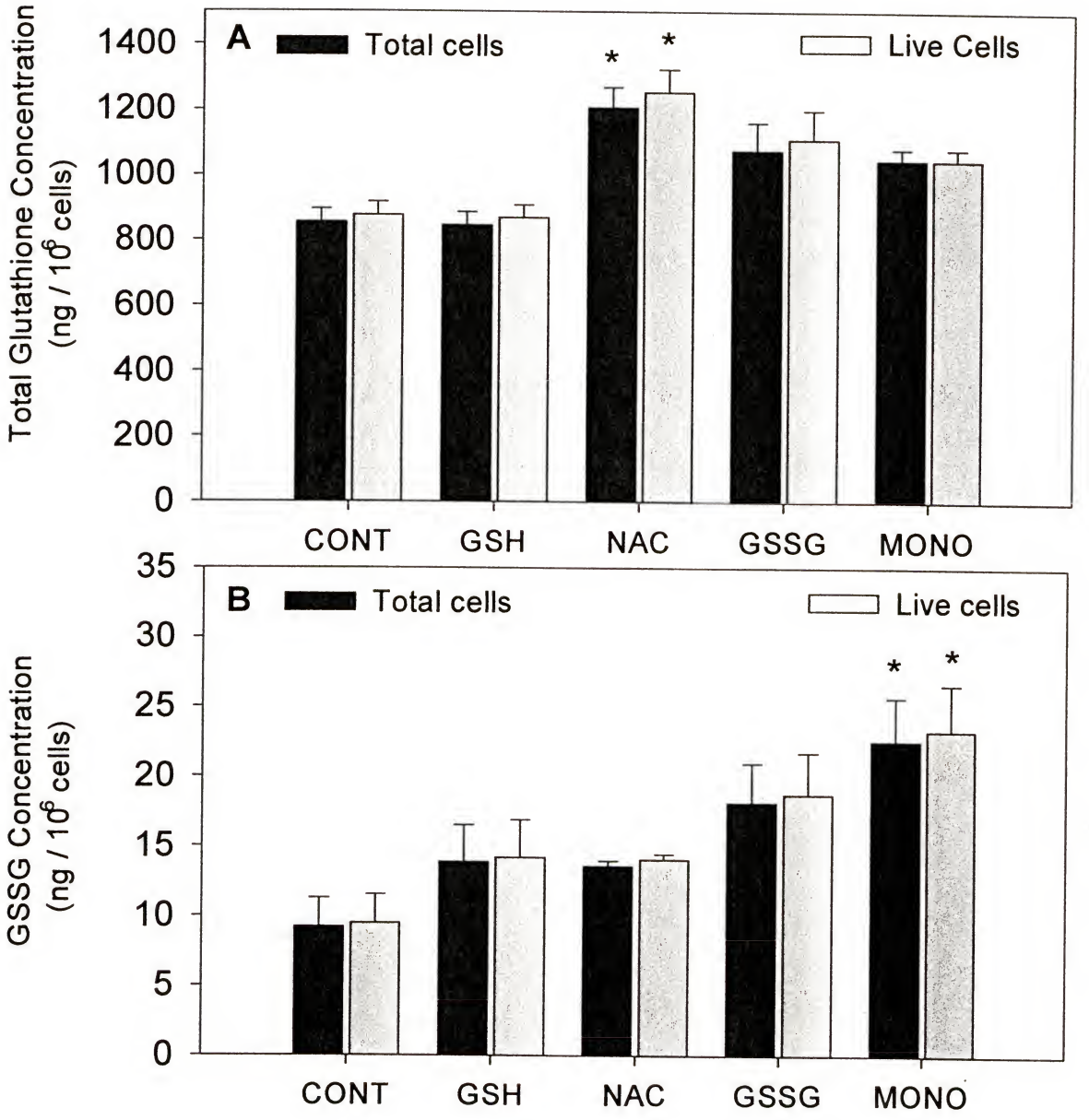

Fig. 7.4 Glutathione concentration in SK-N-SH cells after treatment with drugs known to increase intracellular content. A) Total glutathione content; B) GSSG content. Cells grown in 100 mm dishes (n= 3 dishes per group) subjected to GSH (3.25 μM), NAC (100 mM), GSSG (1.6 μM), or MONO (3.25 μM) treatment for 24 h and assayed for glutathione concentrations. *$p<0.05$ versus the control group as determined by ANOVA followed by Tukey's test post hoc.

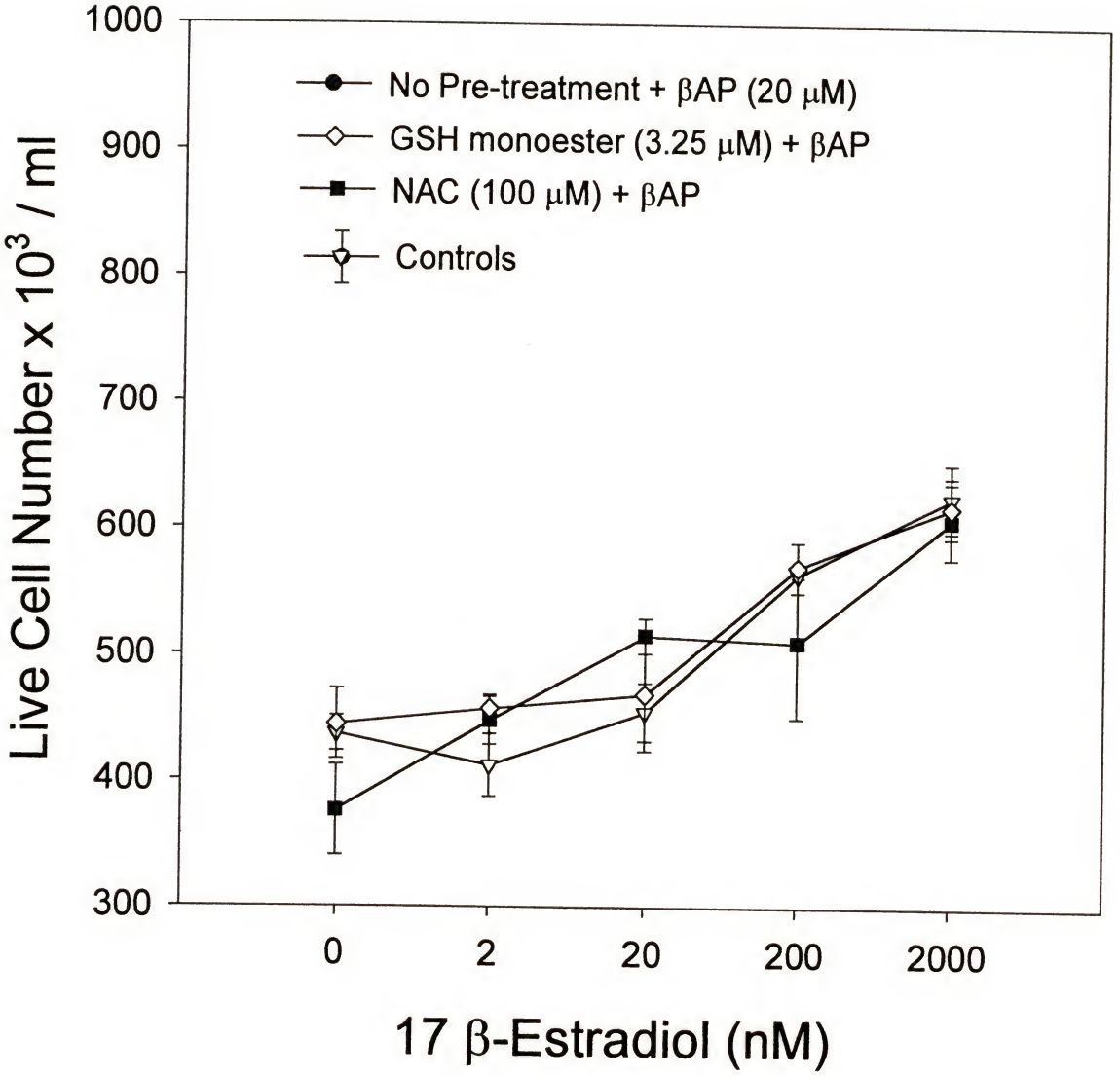

Fig. 7.5. Effects of 24 h of βAP treatment in SK-N-SH cells subjected to increasing doses of E2 after 24 h of pre-treatment with drugs known to increase intracellular glutathione content. Depicted are mean values ± SEM for n = 4 wells per group as determined by trypan blue exclusion. Two way ANOVA revealed no significant differences between groups.

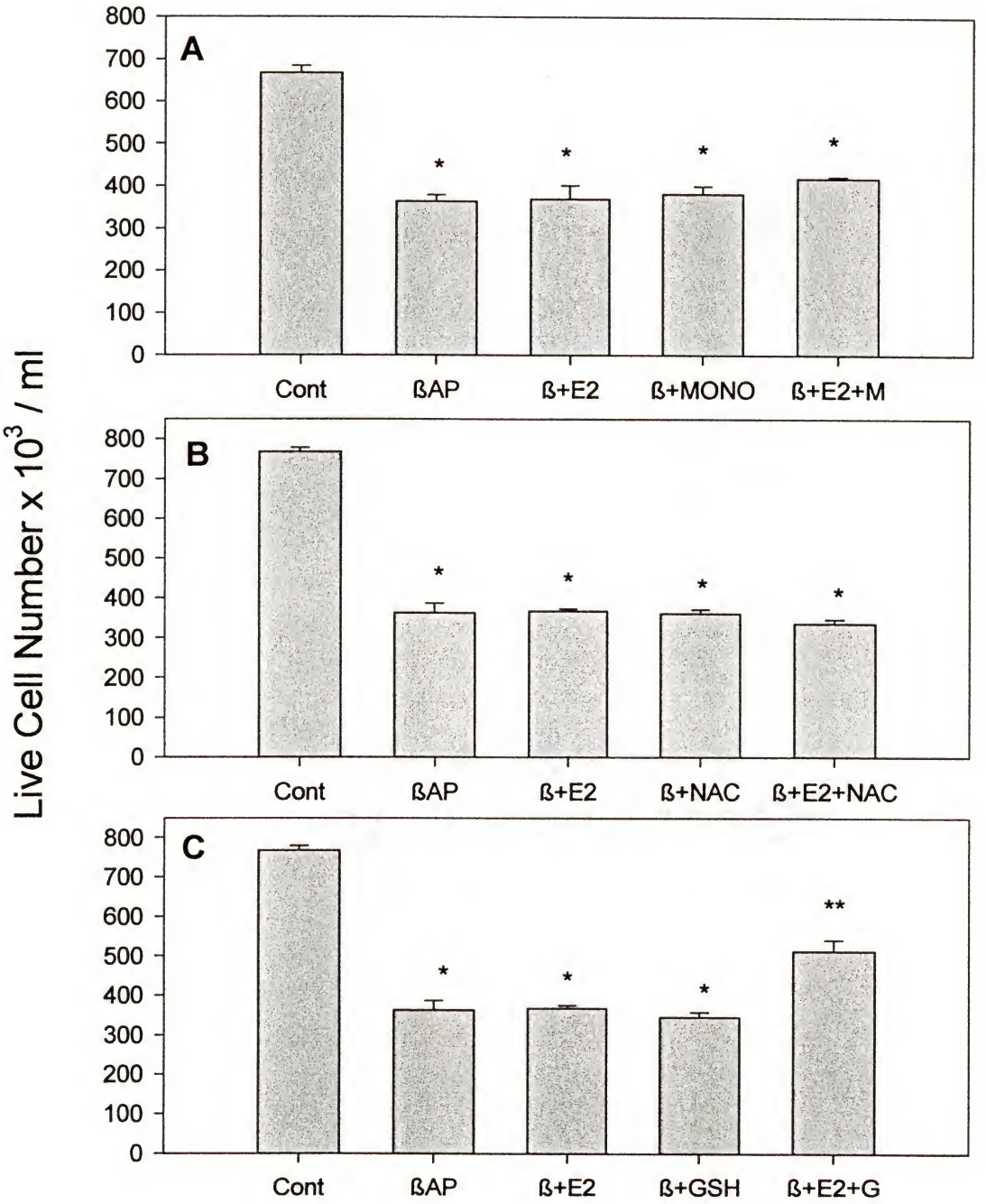

Fig. 7.6. Effects of E2 co-treatment with compounds known to increase intracellular GSH and GSSG on βAP (20 μM) induced toxicity. A) Glutathione monoester (MONO; 3.25 μM), B) n-acetyl-l-cysteine (NAC; 100 μM), and C) GSH (3.25 μM). Cells plated at 10^6 cells/ml and treated as indicated for 24 hours. Represented are means ± SEM for n=4 wells/group. *$p<0.05$ versus controls; **$p<0.05$ versus all other groups as determined by ANOVA followed by Tukey's test post hoc.

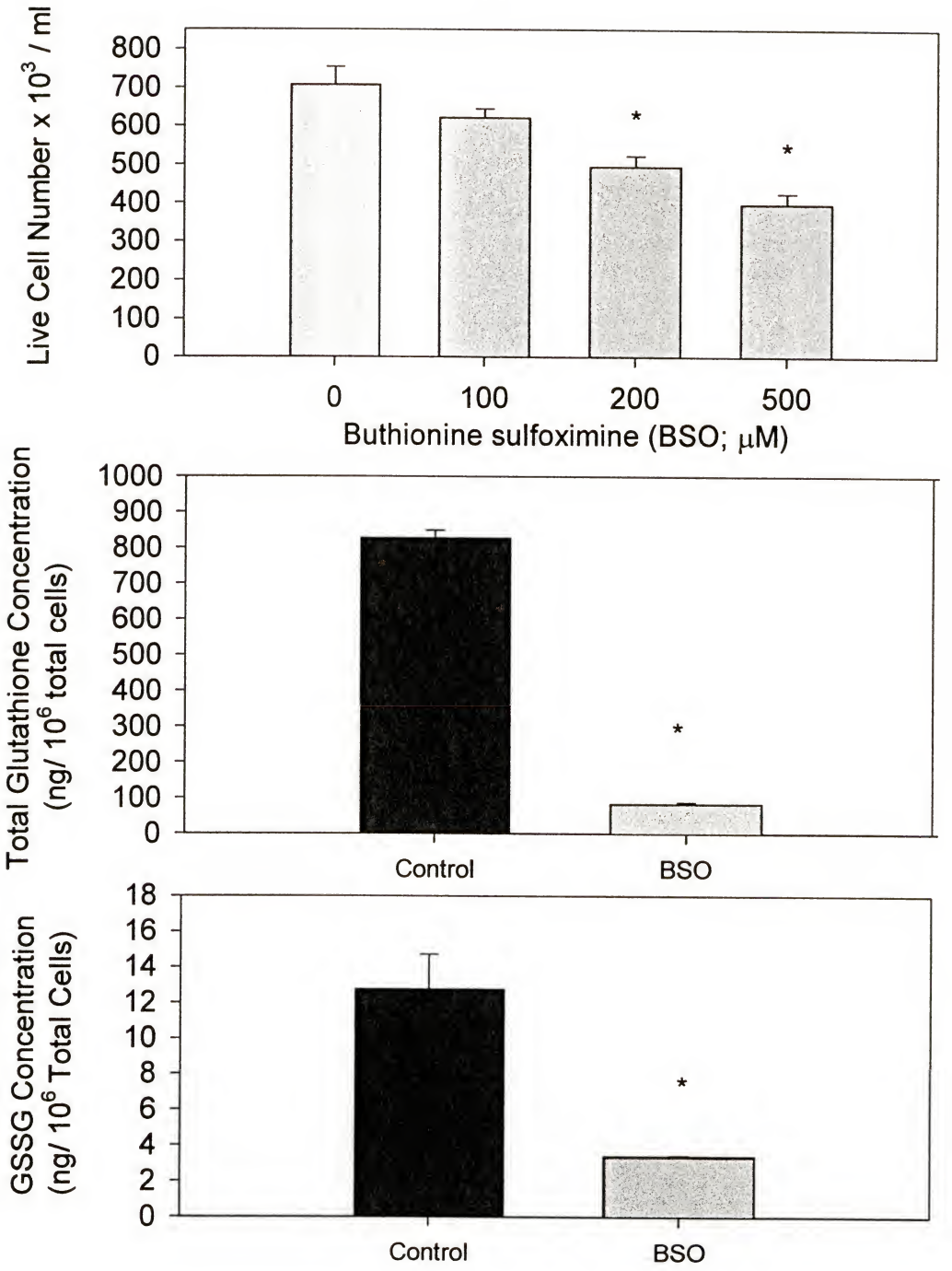

Fig. 7.7. Effects of buthionine sulfoximine (BSO) on SK-N-SH cells. A) Toxicity of BSO at increasing concentrations, B) Effect of BSO (100 μM) on total glutathione concentration, and C) effect of BSO on GSSG concentration. Cells plated at 10^6 cells/well for toxicity studies and in 100 mm dishes for glutathione assay with n = 4 wells or dishes per group. *$p<0.05$ when compared to controls.

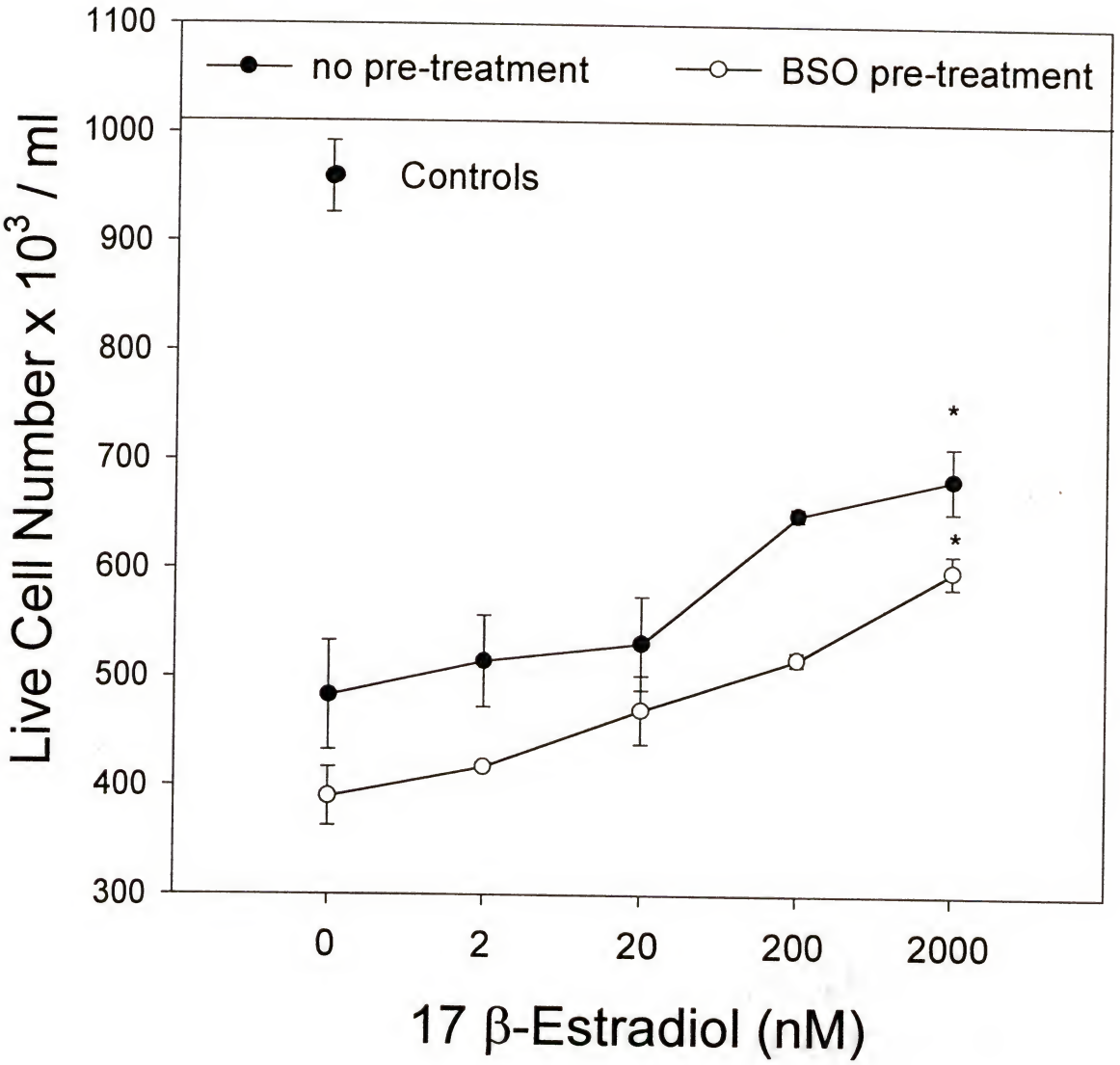

Fig. 7.8. Effects of BSO pre-treatment on E2 dose response to βAP toxicity in the absence of GSH. Cells plated at 10^6 cells per well were subjected to BSO (100 μM) for 24 h and then treated with βAP (20 μM) and increasing doses of E2. A significant effect for BSO treatment was identified ($p<0.001$; $F=32.162$) and the 200 nM dose was effective in saving cells. *$p<0.05$ as determined by two way ANOVA followed by Tukey's test post hoc.

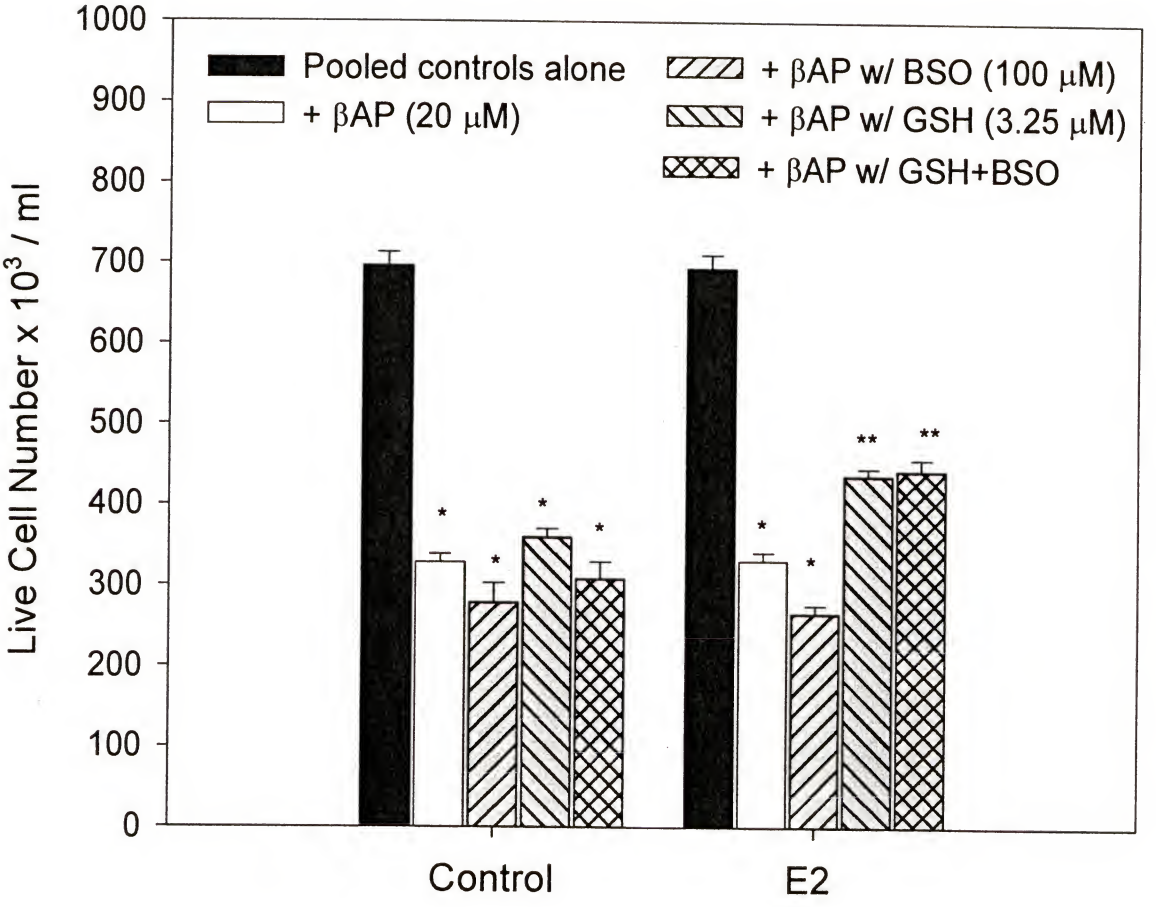

Fig. 7.9. Effect of E2 treatment (2 nM) on SK-N-SH cells subjected to βAP toxicity and co-treated with the presence or absence of GSH (3.25 μM), BSO (100 μM), or the combination as indicated. Depicted is the mean value for live cell number ± SEM for n = 4 wells per group. *$p<0.05$ when compared to controls; **$p<0.05$ when compared to all other groups as determined by three way ANOVA followed by Tukey's test post hoc.

CHAPTER 8
EXAMINATION OF GLUTATHIONE PEROXIDASE AND GLUTATHIONE REDUCTASE ACTIVITIES IN RESPONSE TO BAP TOXICITY IN THE PRESENCE AND ABSENCE OF 17 β-ESTRADIOL AND GLUTATHIONE

8.1 Introduction

Given that the interaction between E2 and GSH does not rely on the cytoplasmic content of GSH and a major role for GSH exists as a substrate for enzymes in oxidation and reduction processes, the possibility exists that the protection afforded by the E2-GSH interaction may result by altering the activity of the glutathione peroxidase (GPX) and/or glutathione reductase (GRD) enzymes. The selenoprotein GPX (E.C. 1.11.1.9) catalyzes the reaction of hydroperoxides to the corresponding alcohol and water by-products by using GSH as a substrate for reducing equivalents with the production of GSSG, the reaction of which was depicted in chapter 3. Because hydroperoxides and lipid peroxides may decompose to highly reactive by-products, the GPX enzymes are crucial to protecting the cell from free radical damage; however, in some instances, GSH S-transferases may exhibit overlap with these reactions. The pool of cellular glutathione is maintained for these purposes with the assistance of GRD (E.C.1.6.4.2), which relies on reducing equivalents supplied by NADPH to regenerate glutathione in its reduced form.

All of the GPX enzymes are tetramers of four identical subunits, each monomer being approximately 22-23 kD. Each subunit has a selenocysteine in the active site which participates directly in electron donation and becomes oxidized in the process. The GSH

substrate is used to then regenerate the reduced form of the selenocysteine. The oxidized glutathione forms a dimer, and is reduced by GRD in multiple steps.

The GRD enzyme exists as a homodimer consisting of four domains beginning at the N-terminus: A FAD-binding domain; a NADPH-binding domain, a central domain, and an interface domain. The dimer surface contains the active sites, and the GSSG binding site is composed of residues from both of the subunits. Initially, oxidized GRD is reduced by NADPH, with the formation of a semiquinone between FAD, a sulfur radical, and a thiol. This reduced GRD subsequently reacts with the GSSG dimer, disulfide exchange occurs, and a molecule of GSH is produced along with the reduced GRD complexed to GS-. Electron rearrangement results in another disulfide exchange, with the generation of the second GSH molecule and restoration of GRD to its oxidized form.

Expression of cellular GPX is present in all tissues, while many other GPX enzymes show tissue-specific expression patterns. GRD is also a ubiquitous enzyme. This chapter is devoted to analyzing the effects of E2, GSH, and the combination in control cells and cells subjected to βAP toxicity to evaluate whether the protection resulting from the E2-GSH interaction affects the activity of these enzymes.

8.2 Materials and Methods

SK-N-SH cells were plated in 100 mm dishes at 4 x 10^6 cells/plate in 10 mls media and allowed to reach 80% confluency. At this time, media was decanted and replaced with the appropriate treatments prepared as stated in earlier chapters, to include vehicle treated controls, βAP (20 μM), E2 (2 nM), GSH (3.25 μM), or a combination as indicated. After 24h of incubation, cells were extracted using the protocol as described in

chapter 3. GRD activity and GPX activity were determined using kits obtained from Calbiochem. Basically, the GPX and GRD activities were measured indirectly based on the oxidation of NADPH to $NADP^+$. The oxidation of NADPH to $NADP^+$ was monitored for a specified time via the absorbance at 340 nm, with the rate of decrease measured corresponding to either the GSH oxidized by GPX, or the amount produced by GRD from GSSG; both correlate to the enzyme activity in the sample. The GPX and GRD activities were calculated using 0.0622 as the molar extinction coefficient for NADPH. The activities were normalized to DNA content, and analyzed for statistical significance using ANOVA.

Viability studies examining the co-treatment of NADPH with E2 studies were performed on SK-N-SH cells by plating 1 x 10^6 cells/ well in 24 well plates in DMEM and incubating for 4 h to allow for cell attachment. At this time, media was decanted and cells were treated as follows: vehicle treated controls, E2 (2 nM), βAP (20 μM), NADPH (3.25 μM) or a combination as indicated, with GSH (3.25 μM) combined with E2 as a positive control.

8.3 Results

The activity of GRD was unaffected by any of the treatments (Fig. 8.1) and was not considered significant by statistical analysis. Likewise, the data for GPX activity mirrors these results (Fig 8.2). When the possibility was considered that GSH may be interacting with E2 to provide neuroprotection by affecting the concentration of NADPH, the substitution of NADPH was made for GSH in combination with E2 and evaluated for increase in live cell number by trypan blue exclusion (Fig 8.3). BAP treatment in this

experiment decreases the live cell number by 55%, and no protection was noted using NADPH or NADPH in combination with E2. A significant increase in live cell number of was seen with the addition of E2 and GSH when compared to βAP treated cells alone and used as a positive control for comparison with other groups.

8.4 Discussion

Exogenous estradiol exerts effects on endogenous antioxidant defense mechanisms, including increasing erythrocyte glutathione peroxidase activity in women [103] and oral contraceptive use has been correlated with an increase in glutathione peroxidase activity [125]. In addition, uterine GPX activity was significantly increased by exogenous estrogen treatment [151]. Hormonal regulation of GSH transport has been established [85] and glutathione peroxidase activity was found to be higher in females than males [94,117]. Taken together, these data suggest that estrogens may increase the activity of this enzyme system.

Increased GPX activity has been attributed to a subclone of cells deemed resistant to βAP toxicity [177]; however, the activities presented here using the SK-N-SH cell culture model are largely unresponsive to βAP toxicity or the combination of E2-GSH treatment. These observations are in agreement with the lack of difference in GSH content between control cells and cells with E2-GSH treatment, as no apparent change in enzyme substrate agrees with no apparent change in enzyme activity. Perhaps the difference between this work and work by Sagara [177] occurs because the total population of cells is examined in these experiments, and not solely the surviving population.

In addition, cellular GPX activity may overlap with that of some glutathione S-transferases, and GPX also has distinct isoforms which are present in plasma [121,204]. Likewise, GRD activity may include some thioredoxin activity as well. Lastly, the measurement of GPX activity has some pitfalls, e.g. enzymatic side reaction of substrates, high and variable spontaneous reaction rates of substrates and some peculiar enzyme kinetics including ping-pong kinetics with numerous limiting maximum velocities and Michaelis constants [62]. These effects were minimized by running blanks for each sample.

GPX activity appears to be unaffected by aging in the rat brain, while the activity of GRD appears to be somewhat decreased [18]. Conversely, the activities of the pentose phosphate shunt enzymes, glucose 6-phosphate reductase and glucose 6-phosphate dehydrogenase, were either increased or unaffected [18]. An imbalance of GPX and GRD in the cell would lead to the reduced GSH pool as seen in aging, and it is interesting that a decrease in GRD activity occurs in spite of increased reducing equivalents [18]. Since these are all changes noted in animal models, and given that the cell culture data is not provocative, it might be more pertinent to examine the response of these enzymes to estrogen treatment in animal models of aging to gain more insight into the E2-GSH interaction.

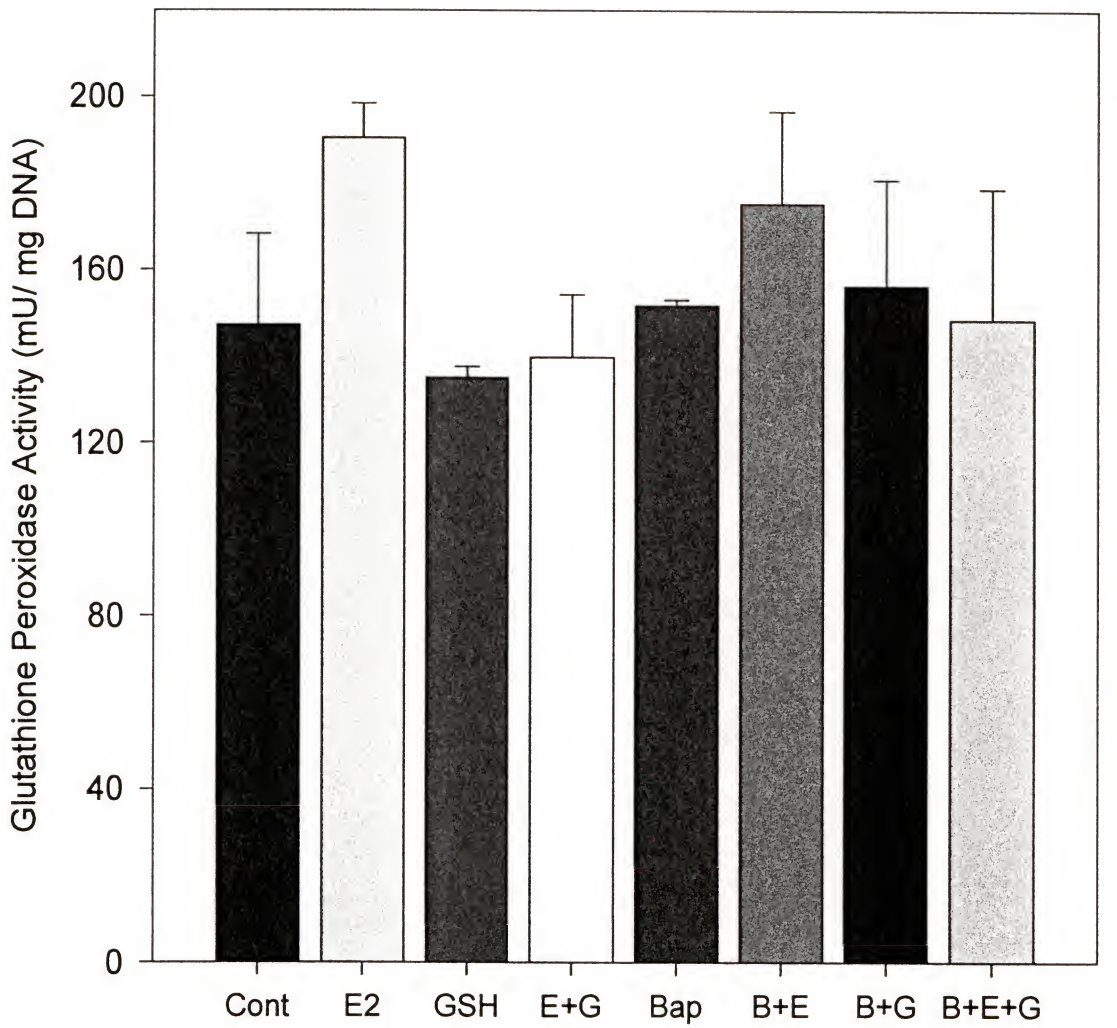

Fig. 8.1. Effects of various treatments on the cellular glutathione peroxidase activity. Cells plated in 100 mm dishes were subjected to E2 (2 nM), βAP (20 μM), GSH (3.25 μM), or the combination as indicated for 24 h. At this time, cytoplasmic proteins were extracted, assayed for activity, and normalized to DNA content. Depicted are mean values ± SEM for n = 3 dishes per group.

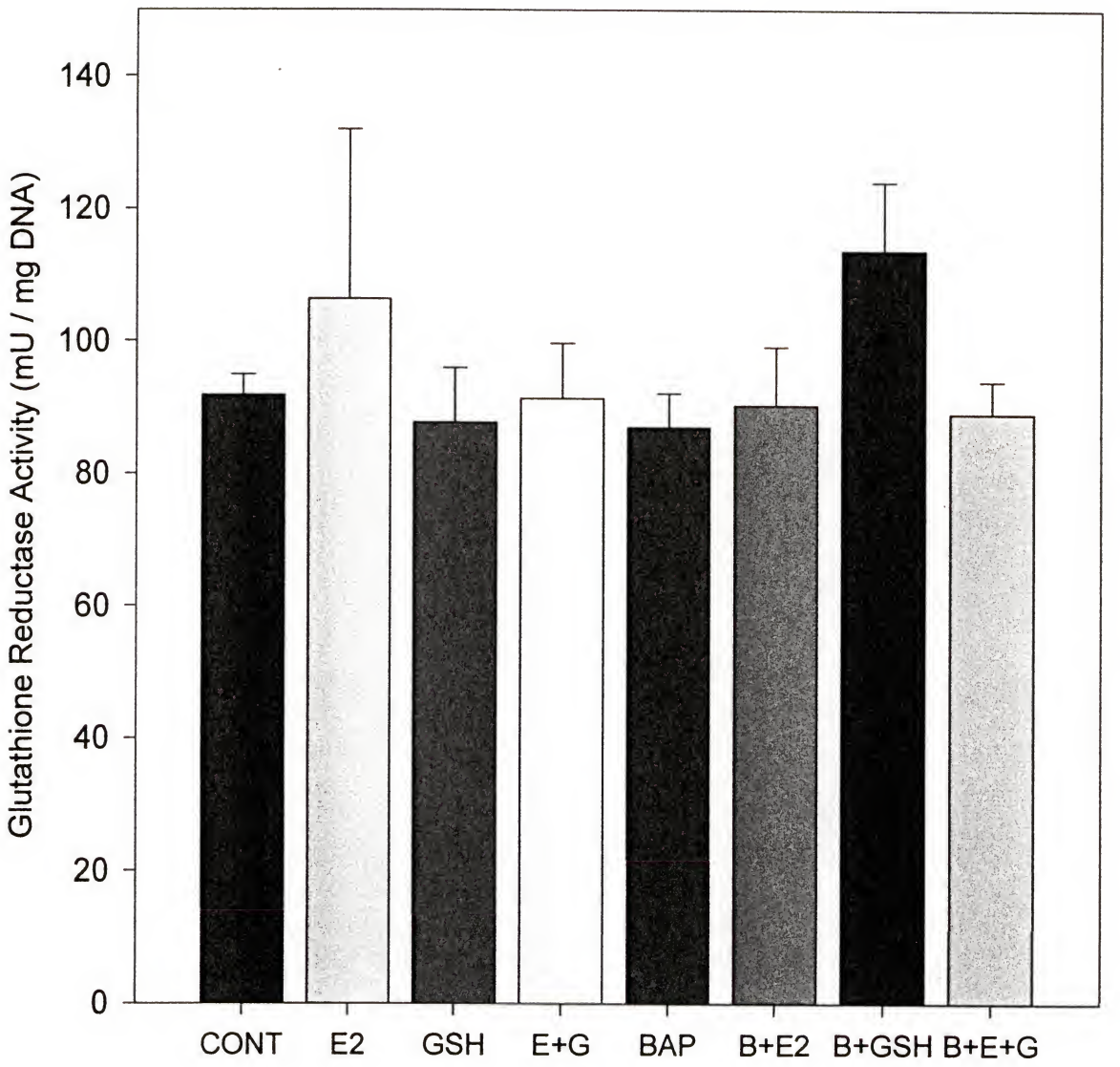

Fig. 8.2. Effects of various treatment on the cellular glutathione reductase activity. Cells plated in 100 mm dishes were subjected to E2 (2 nM), βAP (20 μM), GSH (3.25 μM), or the combination as indicated for 24 h. At this time, cytoplasmic proteins were extracted, assayed for activity, and normalized to DNA content. Depicted are mean values ± SEM for n = 6 dishes per group.

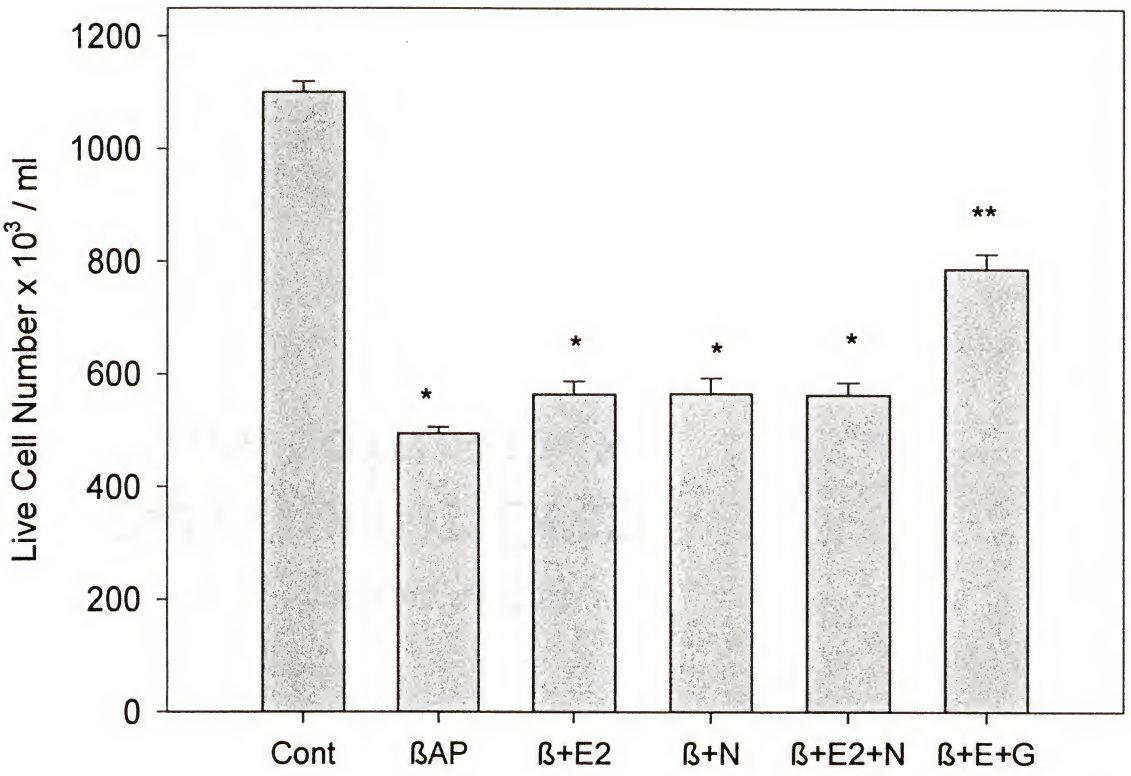

Fig. 8.3. SK-N-SH cells subjected to βAP toxicity (20 μM) and the presence or absence of E2 (2 nM), NADPH (N; 3.25 μM), GSH (G; 3.25 μM) or the combination as indicated. Cells were plated at 106 cells/well and incubated for 24 h. Depicted are mean values for live cell number ± SEM for n = 4 wells per group. *$p<0.05$ versus control; **$p<0.05$ versus all other groups as determined by ANOVA followed by Tukey's test post hoc.

CHAPTER 9
PRELIMINARY STUDY OF THE EFFECTS OF OVARIECTOMY AND ESTROGEN REPLACEMENT ON HIPPOCAMPAL GLUTATHIONE CONTENT

9.1 Introduction

Neurodegenerative conditions ameliorated by estrogen replacement therapy in women are associated with oxidative stress. Glutathione plays a defensive role against oxidative stress although more study is indicated regarding the status of antioxidant defenses in AD patients. *In vivo* interactions between E2 and GSH in humans are substantiated by reports of increased glutathione levels in breast tissue during the mid-luteal phase as compared to the follicular phase at a time when E2 levels are increased from 94 pg/ml to 210 pg/ml [42]. Likewise, oral contraceptive use has been correlated with an increase in glutathione peroxidase activity [125]. In rat models, hormonal regulation of GSH transport has been established [85], and glutathione peroxidase activity was found to be higher in females than males [94,117].

While the protective mechanism between E2 and GSH in cell culture appears to be independent of intracellular GSH content and GPX/GRD activity, the mechanism behind possible E2/GSH interactions in animal models may operate quite differently. The E2/GSH interactions *in vivo* may be identified by accurately measuring glutathione levels in animal models of estrogenic and hypoestrogenic states. This preliminary study was done to assess whether glutathione levels in the hippocampus of rats were affected by hormonal status.

9.2 Materials and Methods

Adult female Sprague-Dawley rats (200-225g) were purchased from Charles Rivers Laboratories (Wilmington, MA) and were maintained for 1 week before female rats were subjected to ovariectomy (OVX). All rats were allowed free access to food and water throughout the procedure. Female rats, anesthesized with Metafane inhalant (methoxyflurane, Pitman Moore, Crossings, NJ) had both ovaries removed through bilateral abdominal incisions using the dorsal approach. For rats receiving sham surgery, skin incisions were made, but the ovaries were not disturbed. After recovering from anesthesia, the rats were returned to their cages.

Estrogen replacement in estrogen treated groups (OVX + E2) was done using silastic implants (Dow Corning, Midland MI). Pellets were made by packing 3 mg crystalline 17 β-estradiol into 5-mm Silastic tubes and sealed on both ends with silastic adhesive. The capsules were washed twice with ethanol in scintillation vials, dried, and placed in saline solution for 24-48 h before implantation. Sham animals received cholesterol containing controls. The pellets were implanted subcutaneously in the animals and the animals were housed in the animal care facility for either 4 or 8 weeks, with decapitation occurring at that time.

Brains removed from decapitated rats were dissected, and the hippocampal tissue excised. At this time, tissue was weighed, homogenized in 1% sulfasalicylic acid, diluted to 1 ml in buffer, and maintained on ice in tightly capped conical tubes to help minimize auto-oxidation of GSH. The conical tubes were spun in the centrifuge on 8000-10,000 g at 4° C for 15 minutes. Aliquots were stored at -80° C until assayed for GSH and GSSG

as previously described. Statistical significance for the effects of time of ovariectomy and estrogen treatment were determined using two way ANOVA, and significant interactions analyzed using Tukey's test post hoc.

9.3 Results

No detectable difference was observed for the presence or absence of E2 treatment for OVX, OVX+E2, or sham animals with regard to the total glutathione content (GSH+GSSG) of hippocampal tissue (Fig. 9.1). Likewise, the duration of treatment had no effect on glutathione content, as 4 week groups were not different from 8 week groups (Fig. 9.1). This is contrasted by the GSSG content of the samples (Fig. 9.2), in which two way ANOVA reveals a significant effect ($p<0.001$; $F=11.583$) of E2 treatment when compared to OVX and SHAM operated animals. In E2 replaced animals, the GSSG content is doubled when compared to sham animals at both time points (Fig. 9.2). Likewise, OVX + E2 animals show a 7.5 fold increase in GSSG at the 4 week time point when compared to the OVX group, which decreases to a 3.4 fold increase by the 8 week time point.

Because the ratio of reduced to oxidized glutathione may be important in establishing the presence of oxidative stress in tissues, the amount of reduced glutathione was calculated by subtracting the GSSG value from the total glutathione content of the sample and subsequently ratios of reduced to oxidized glutathione were analyzed (Table 9-1). It would follow that due to the increased GSSG content of the sample, the ratio of GSH:GSSG decreased in a similar fashion. When the data was analyzed for change in electrochemical potential, calculated using the Nernst equation and the calculated values

for GSH and measured values for GSSG as discussed in Chapter 7, no significant difference was detected (Table 9-1). Estrogen values for each group are also listed for comparison.

9.4 Discussion

The major question to be addressed is whether the increase in GSSG content in the E2 treatment group is physiologically relevant. On first glance, one might think that an increase in GSSG levels would indicate a higher level of oxidative stress as reduced GSH is oxidized in the presence of free radicals. It seems counterintuitive to associate this increase with the E2 treated group. This is compounded by the ratio of GSH:GSSG for the E2 treated animals, which demonstrate differences as well. Although this is used widely as an indicator for oxidative stress, it may not be an appropriate index. The electrochemical potential, showing no difference in treatments, may indeed be representative of the cellular redox state.

One major flaw associated with this study was the amount of time for storage of tissue, which could not be helped due to technical difficulties experienced with the Tietze assay in the time frame of these experiments. Steps taken to minimize autooxidation of GSH to GSSG included storage of the samples in acid, and it is unlikely that autooxidation only occurred in one group. More likely, the GSSG content of the samples would all be artificially increased.

Because the data presented indicate an effect of estrogen replacement, future studies should be completed. Handling of samples should be given the utmost attention. Effects on other parameters, such as GPX, GRD, GSH and GSSG content of a number of

brain regions, as well as CSF, red blood cells and blood plasma are all important to understanding the physiology behind an E2-GSH interaction in the brain.

Generalized oxidation, demonstrated by a decline in the reduced GSH pool and an increase in the oxidized pool, has been associated with aging in males [178], and male rats [19]. Whether the cerebral GSH system is modified by metabolic events rather than by circulatory events still remains in question [19]; yet, these experiments have only been carried out in male subjects. Given the results presented here, similar experiments carried out in females should be a priority.

Table 9-1. Blood estrogen levels and hippocampal GSH:GSSG ratios and redox potentials of female rats subjected to ovariectomy (OVX), ovariectomy with estrogen replacement (OVX+E2), and sham operated controls (SHAM) for either four or eight week time periods. N=4 for 4 week groups with regard to the ratio and potential data; n=8 for all 8 week groups and all E2 levels, mean values listed with (SEM).

TREATMENT	E2 Level (pg/ml)	GSH:GSSG ratio	Redox Potential
SHAM–4 weeks	16.19 (2.49)	144.8 (66.1)	-337.3 (33.0)
OVX–4 weeks	12.92 (1.37)	377.8 (86.7)	-383.8 (4.5)
OVX+E2–4 weeks	23.72 (1.26)	66.3 (25.6) *	-361.8 (4.9)
SHAM–8 weeks	12.26 (0.84)	327.5 (61.9)	-378.8 (3.8)
OVX–8 weeks	9.56 (0.80)	371.9 (76.9)	-381.5 (3.1)
OVX+E2–8 weeks	29.05 (11.02)	122.1 (23.1) *	-371.0 (4.0)

*A significant effect of estrogen treatment was noted ($p=0.001$; $F=8.667$) using two-way analysis of variance.

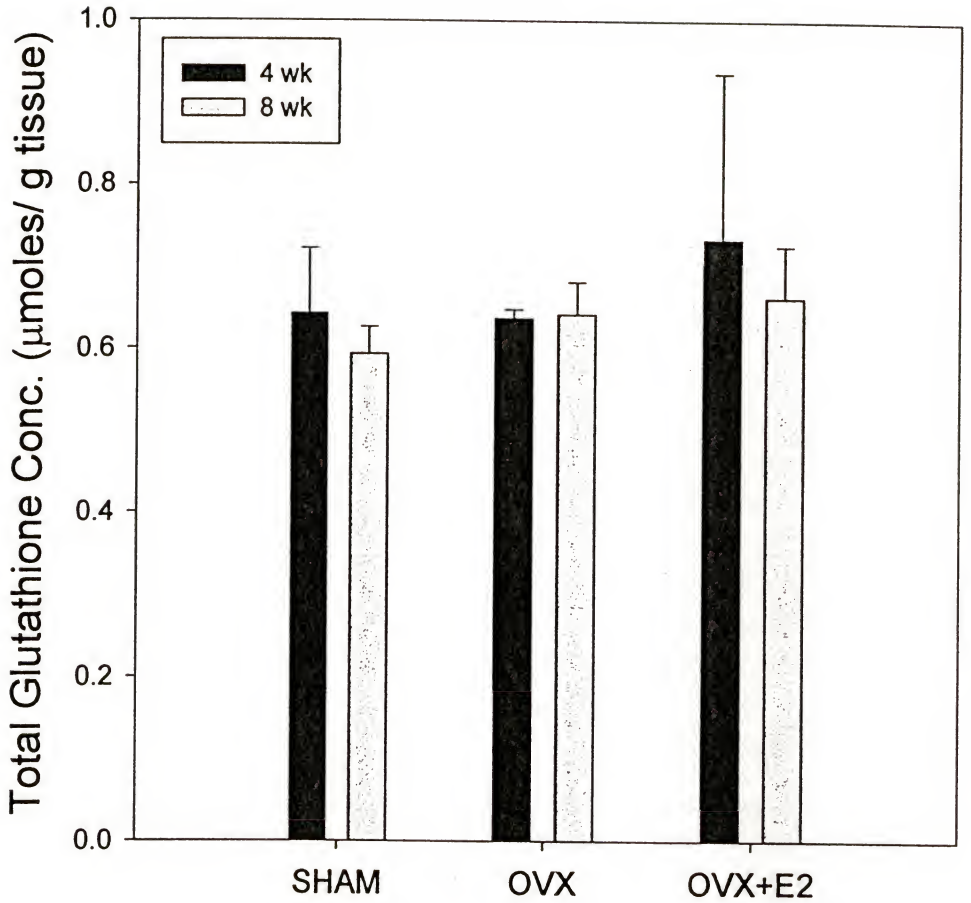

Fig. 9.1. Total glutathione concentration (GSH+GSSG) in the hippocampus of female rats undergoing ovariectomy (OVX), OVX with E2 replacement, or sham operations for either 4 weeks (n = 4) or 8 weeks (n = 8) of treatment.

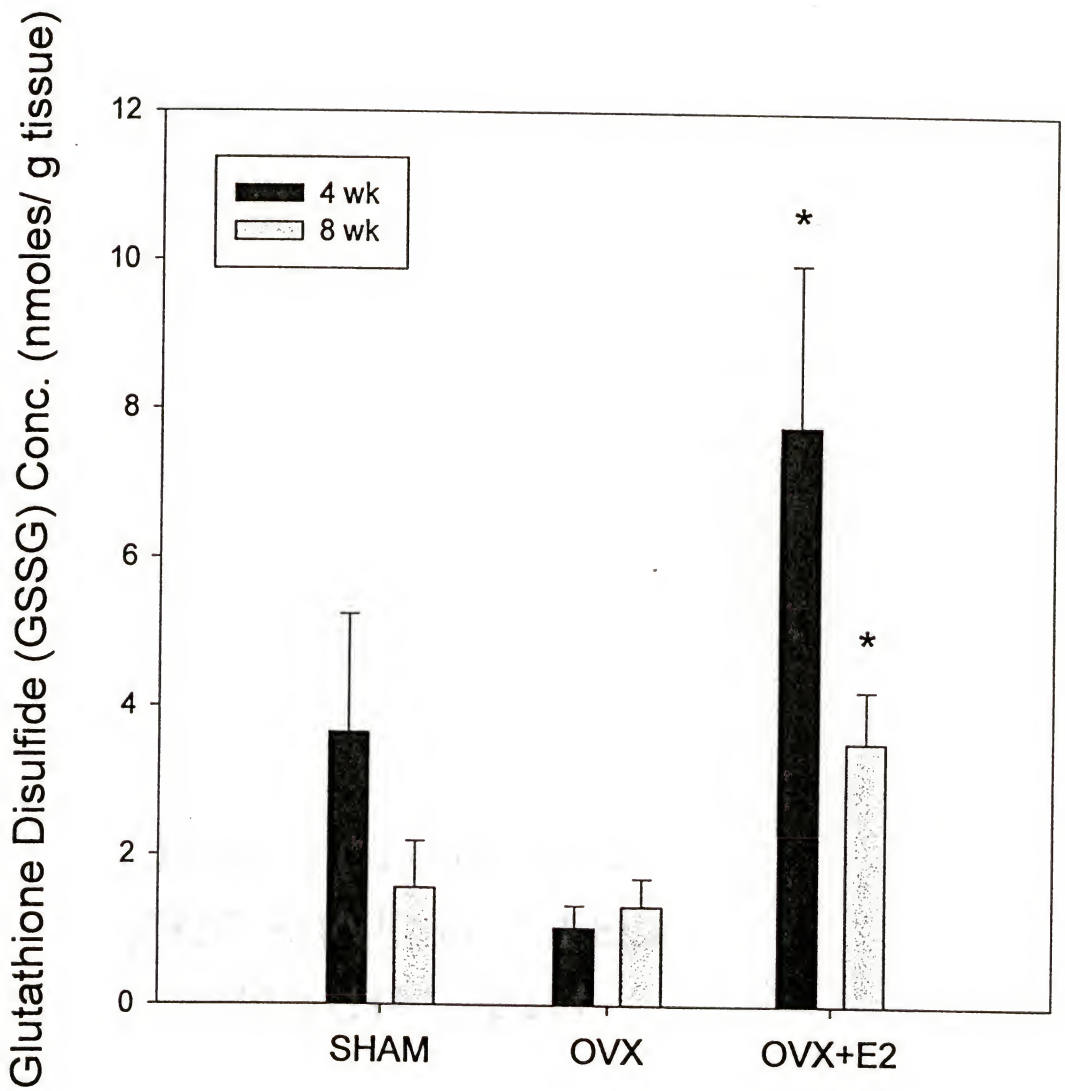

Fig. 9.2. Glutathione disulfide concentration (GSSG) in the hippocampus of female rats undergoing ovariectomy (OVX), OVX with E2 replacement, or sham operations for either 4 weeks (n = 4) or 8 weeks (n = 8) of treatment. *$p<0.001$, $F=12.077$ showing a significant effect for treatment as determined by two way ANOVA followed by Tukey's test post hoc when compared to all other groups.

CHAPTER 10
SUMMARY AND NEW DIRECTIONS

This is the first report of a novel synergistic interaction between E2 and GSH in neuroprotection. The mechanism of this interaction remains yet a mystery. Likewise, the physiological relevance for this interaction is debatable. Support for the idea that this interaction is relevant is demonstrated by the block of this synergy with progesterone, and the range of cell types and insults where synergy can be replicated.

10.1 Physiological Relevance

One major question for relevance is whether the concentrations of estrogen which provide protection in these experiments can be compared to what is obtained therapeutically with a postmenopausal hormone replacement regimen. The normal range for estradiol in cycling women is between 50-200 pg/ml [48]. In a study of postmenopausal women with AD, the circulating E2 levels were reported to be 2-6 pg/ml prior to estrone sulfate treatment [92]. After 3 weeks of this treatment with estrone sulfate, which is a major component of Premarin®, the circulating estradiol levels increased to 13-21 pg/ml. After 6 weeks of treatment, levels increased to 26-76 pg/ml [92]. Cognitive testing performed at both of these time points resulted in 5 out of 7 women experiencing benefits from hormone replacement, which correlated to an increase of 7-19 pg/ml of circulating estradiol [92]. However, it is likely that the total estrogen concentration (including estrone and conjugated estrone with estradiol) is much higher.

Relating this to a cell culture model may require some assumptions. First, assuming no binding of estrogen, the total free amount of estradiol in the SK-N-SH cell culture model would be 544 pg/ml at the 2 nM dose, and 54 pg/ml at the 0.2 nM dose. The 0.2 nM dose is the lowest dose which in the presence of GSH shows neuroprotection [77]. Quality control studies, performed while monitoring the stability of estradiol compounds in solutions of absolute ethanol, showed a calculated solution of 1 μM E2 to actually be 0.43 μM upon testing with radioimmunoassay (personal observation). Based on this percent recovery, 23 pg/ml would be the amount of estrogen participating in neuroprotection. This concentration is certainly in proximity to the range calculated from the data presented by Honjo.

The component of free or bound estrogen which is responsible for the cognitive benefits associated with estrogen replacement therapy is another question. Assuming that 98% of the estrogen circulating was bound to sex hormone binding globulin, and that free estrogen was responsible for the cognitive benefits, the calculated active fraction of circulating estradiol would be approximately 1.52 pg/ml at the highest level reported by Honjo. If all of the estrogen in this cell culture model were free, then the level seen by the SK-N-SH cells is approximately 15 times higher; however, this seems unlikely based on the percent loss described above. Cell culture media contains 10% fetal bovine serum, and although it is charcoal stripped to ensure non-detectable levels of steroid hormones, plasma proteins are still present which could participate in non-specific binding of estrogens. Likewise, non-specific binding could occur with all of the plastic products used during cell culture, including pipette tips, microcentrifuge tubes and culture dishes. Thus,

it is likely that the bound E2 levels could reach the same percent fractions as those seen in plasma, and that the free concentrations used in these cell culture experiments would mirror the human data.

The physiological relevance for the GSH dose also deserves discussion. The intracellular level of GSH in mammalian cells is 0.5 - 10 mM, whereas micromolar concentrations are typically found in the blood plasma and extracellular environments [133]. The data herein support the conclusion that the cytoplasmic content of GSH is not involved. Likewise, the concentrations of GSH used in these experiments which provide protection are at levels found in the extracellular environment. As such, this raises the question of whether the reported synergistic interaction between E2 and GSH is occurring in the extracellular space.

10.2 Summary of Possible Mechanisms

Several possibilities exist which could explain the observed synergistic interaction between E2 and GSH (Fig. 10.1). Receptor mediated actions are the classic mechanism for estrogen associated effects; however, effects attributed to GSH are rarely considered to be receptor mediated. It is a possibility that both E2, given the recent discovery of different receptor subtypes, and GSH [185] act on receptors. The estrogen receptor involved in this effect may act through a mechanism independent from the classical estrogen receptor. The data obtained using the ICI compound support this idea, but direct effects of the ICI compound as an active agent due to its phenolic A ring cannot be ruled out. In addition, membrane bound E2 receptors exist, but conjugation of estradiol with bovine serum albumin at the 17- or 6- carbon positions [78], which prevents the

appropriate orientation of the molecule into the plasma membrane, blocks the neuroprotective action of estradiol. GSH has been discussed as a possible neurotransmitter and neuromodulator [185]. Given this information, the synergy could occur at a convergence point for these possible receptors and provides one possible, even though I believe unlikely, explanation.

The protection afforded by the E2-GSH interaction does not appear to be correlated with cytoplasmic GSH status. Three lines of evidence support this idea: 1) SK-N-SH cells are still protected by the E2-GSH combination in the presence of βAP and a well-known GSH depleting agent; 2) compounds which increase the intracellular GSH have no neuroprotective effects when given under pre-treatment or co-treatment conditions with E2; and 3) the GSH content of cells is unaffected by E2-GSH treatment in the presence or absence of toxicity.

The remaining sites to be considered for a possible interaction include the mitochondria, the nucleus, and the extracellular space. While more research is necessary, the mitochondria is the least likely to be involved of these choices although it cannot be ruled out based on my experiments. AD has been correlated with mitochondrial gene mutations and metabolic disturbances. Given that 20-30% of the cellular mitochondrial pool resides in the mitochondria in some cell types [196], BSO leaves the mitochondrial pool intact [83], and estrogens stabilize mitochondrial function [126], the possibility does exists that the E2/GSH synergy could function to protect mitochondria against oxidative damage. Perhaps estrogens interfere with the mitochondrial permeability transition, or

block release of mitochondrial proteins such as cytochrome c which could participate in the apoptotic cascade.

Arguing against a mitochondrial mechanism is the low dose of GSH which is needed for this interaction with E2. Thirty percent of the cytoplasmic pool of GSH is still 150 μM (at the low end of the reported range), and the addition of 3.25 μM GSH would seem insignificant compared to this amount. Another piece of information which argues against the mitochondria is the lack of protection afforded by the GSH monoester compound. The monoester compound has an intact thiol group, is esterified on the glycine carboxyl group, and is transported into the cell much faster than glutathione itself [2]. The monoester is converted into GSH intracellularly [2], and the increase in GSH could then be transported into the mitochondria. Transport of GSH from the cytoplasm into the mitochondria turns over approximately every two hours [83]. The 24 hour incubation period in these experiments is certainly long enough to allow for this type of interaction; however, GSH monoester was not able to protect cells in the presence of E2 either when pre or co-treated. Perhaps more interesting is the idea of a structural requirement for GSH which is blocked by the addition of the monoester. E2 structural requirements for neuroprotection have been well defined in a serum deprivation model and include the presence of an intact hydroxyl group [15,76] and at least three rings of the steroid molecule [76]. If the synergy occurred between the two molecules, the structural requirement would be paramount.

An increasingly popular idea in the arena of oxidative stress is the participation of oxidized and reduced molecules in cell signaling cascades. Likewise, gene expression is

sensitive to redox state [1]. Given the phenolic nature of estrogens, they could activate the antioxidant response element/electrophilic response element (ARE) [140]. The ARE has been shown to be activated by phenolic antioxidants to increase phase II enzymes, which includes GSH S-transferase [97]. Thus, a consideration of conjugation of glutathione to estrogens [99] should be addressed. This action would proceed via glutathione S-transferases, where conjugation on the 4 position of the A ring of the steroid molecule provides bulky sustituents [52], which have been shown *in vivo* to greatly increase the antioxidant potential [136]. Likewise, if estrogens were oxidized, they would form quinones, which could also activate the ARE and result in the production of quinone reductase [97]. If the reduction of the estrogen quinone involved the use of GSH as a substrate, another explanation for synergy would exist. The protection of these enzymes against oxidative stress resulting from increased quinones would occur upstream from the mitochondria, and would likely not depend on increased cytoplasmic GSH concentrations for protection. The enzyme activities of GRD and GPX are largely unresponsive to the combination of E2-GSH treatment, however, all of the isoforms of GPX were not considered [121,204] including plasma isoforms, which may lend support to the idea of an extracellular mechanism.

On a cellular level, lipophilic estrogens that partition to the plasma membrane should associate their phenolic A rings with the charged hydrophilic head groups of the membrane phospholipids. Based upon the observation that βAP aggregates in the extracellular space and causes membrane lipid peroxidation [16,71,79], a likely prediction could be the hydroxyl hydrogen of estradiol is donated to prevent the cascade of

membrane lipid peroxidation. Additionally, the enhanced potency of estrogens may result from its ability to donate hydrogen ions from several positions on the A ring [98]. One oxidized form of estradiol which could result from this hydrogen ion donation is the estrogen quinone. Given the state of flux of the plasma membrane, if the oxidized estrogen were extruded from the plasma membrane to the intracellular space, perhaps cell signaling cascades, such as the ARE, are activated. If the oxidized estrogen is extruded from the membrane into the extracellular space, then plasma glutathione peroxidases exist which might repair the estrogen using GSH as a substrate, and thus provide an extracellular mechanism for protection. Both mechanisms may ultimately be participating in the identified synergy.

Given that an extracellular E2/GSH interaction is a possibility, future directions should include investigating the contribution of GSH by glia to the extracellular milieu, and whether this GSH could interact with E2. The physiological relevance of an extracellular mechanism would certainly be strengthened by describing this type of cooperation between cell types. Glia comprise a substantial proportion of brain mass, and interactions between glia and neurons exist in such areas as glutamate reuptake. Glia also serve a nutritive role for neurons and are paramount in the making of myelin. Likewise, evidence exists that astrocytes are less vulnerable to reactive oxygen species than neurons [115], and biochemical and histochemical data point to significant GSH levels in astrocytes [23]. Since glutamate is a constituent of GSH, GSH production and release into the extracellular fluid by glia would replicate the addition of GSH into the cell culture media in these experiments.

10.3 E2 Dependence on GSH for Protection

The dependence on GSH for E2 protection in most, but not all of the insults tested, would suggest more than one mechanism for cell death in the toxicities used. In neurodegenerative diseases, there is a loss of particular subsets of neurons, which may be a consequence of the type of neuronal death which occurs. The type of death may be attributable to a variety of cellular insults, occurring singly or in combination, and may include such events as excitotoxicity and/or oxidative stress. Necrosis is caused by traumatic events with a rapid collapse of internal homeostasis [24] characterized by the selective loss of membrane permeability, resulting in the swelling of organelles and subsequent rupture of the plasma membrane [190]. By contrast, apoptosis is a programmed cell death characterized by cell shrinkage, membrane blebbing, and genomic fragmentation [53].

My data suggest that synergy between GSH and E2 prevents apoptotic cell death (Fig. 10.1). The insults in which GSH plays a role in protection, like βAP and serum deprivation, are associated with apoptotic cascades [66,119] and oxidative stress may participate in apoptotic signaling [57]. On the other hand, zinc/glutamate toxicity may cause a necrotic type cell death, in which the presence of estrogen either stabilizes the plasma membrane or blocks channels which are necessary for cell death to occur. Certainly, glutamate has been associated with excitotoxicity, and high concentrations of glutamate and other excitatory amino acids selectively kill neurons by their depolarizing actions [152]. In the absence of excitatory amino acid stimulation, disturbances of intracellular Ca^{2+} homeostasis may also result in the alteration of cell function, cell

blebbing and lysis [154]. The toxicity of Zn^{2+}, given its cationic charge, could function to disturb the Ca^{2+} balance in the presence and absence of excitatory amino acid stimulation and produce both types of cell death.

The free radical theory of aging postulates that generalized oxidative stress is responsible for aging. Certainly, the neurodegenerative diseases discussed herein have age as a risk factor. Generalized oxidation has been associated with aging in males [178], and male rats [19]. The reduced GSH pool goes down with aging and the oxidized pool goes up, and it may be that the cerebral GSH system may be modified by metabolic events rather than by circulatory events [19]. Yet, these experiments have only been carried out on males. Females on the average live 6 years longer than men [3], and it could be that the interaction between E2 and GSH is in part responsible for preservation of these pools for longer amounts of time in women. Serum antioxidants such as vitamin E also decline with advancing age [213]. The emerging view that metabolic events in the mitochondria are important in the development of age related diseases makes the decline in antioxidant defense even more critical. In females, the decline in estrogen as occurs at the menopause is another example of a compromised antioxidant defense. Identifying the physiological role for the E2-GSH interaction may help ameliorate this decline. Further, the role of depleted GSH in disease states such as AIDS and respiratory distress makes this phenomenon applicable to more than just neurodegenerative diseases.

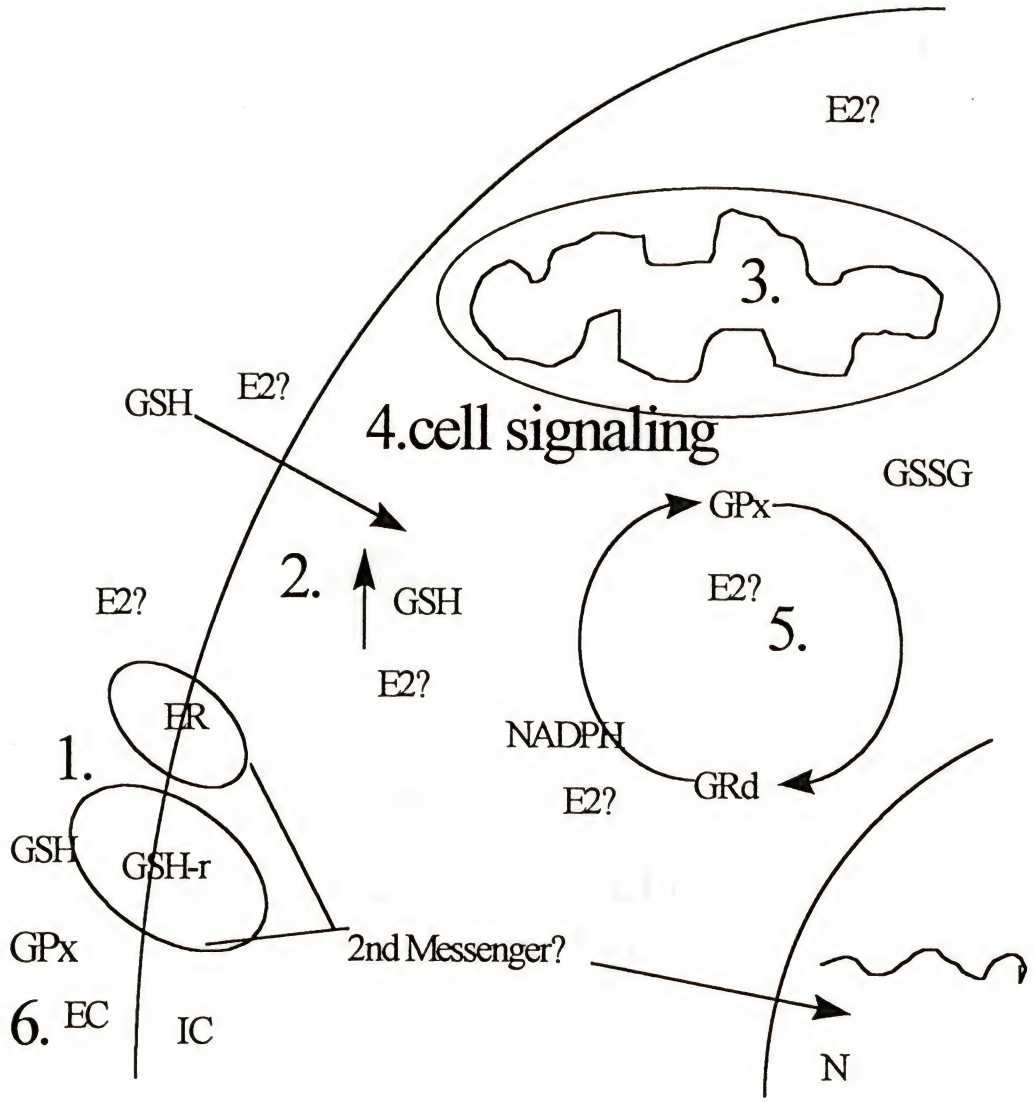

Fig. 10.1. The possibilities for interaction between E2 and GSH. 1) Receptors for GSH and E2 are activated by their ligands and converge to exhibit synergy. 2) Synergy achieved due to increases in the intracellular content of GSH as a result of E2 and GSH treatment. 3) E2 and GSH protective effects exerted on the mitochondria. 4) Oxidized and reduced forms of E2 and GSH activate cell signaling cascades which participate in neuroprotection. 5) Enzyme activities of glutathione reductase and glutathione peroxidase enhanced by the E2/GSH interaction make this enzyme cascade more efficient in protecting against oxidative stress. 6) Extracellular mechanism for protection perhaps involving other cell types.

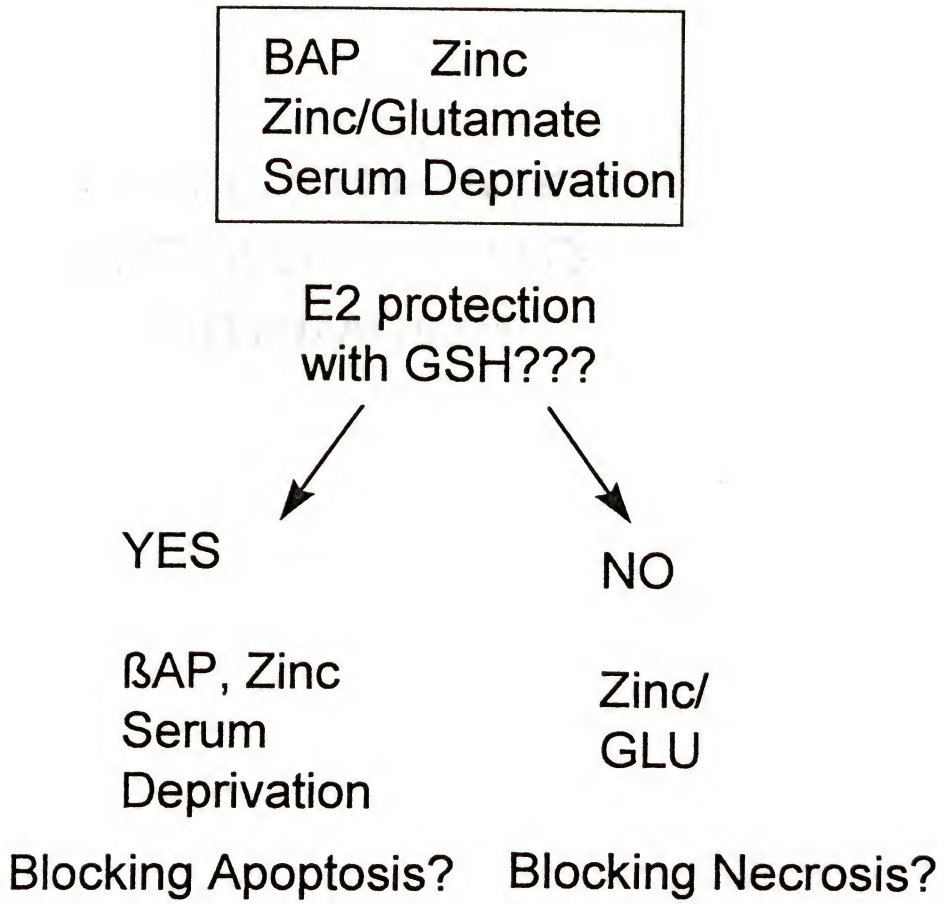

Fig. 10.2. Insults tested in which the dependence for GSH for neuroprotection has been established. Dependence on GSH for protection could indicate insults in which the cell death is more apoptotic in nature, while a lack of dependence on GSH for protection may indicate a more necrotic cell death.

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BIOGRAPHICAL SKETCH

I remember the veneration I felt when I visited the University of Florida for the first time, it was in the summer of 1982. How could I have known that I would spend literally half of my life on this campus? Now, the math is simple: I was born on October 15, 1965, which makes me a 16 (almost 17) year old freshman. I remember Marshall Criser because I shook his hand when I received my Bachelor's degree; now as a graduate student I pay fees in a building bearing his name. And I am 33 years old, writing this biographical sketch for my dissertation.

My graduate school career has been littered with some really lousy personal events. I have had foot surgery, hand surgery, bursitis and torn cartilage in my knee, a concussion, and pneumonia. I have broken off an engagement, been the defendant in a lawsuit, totaled my car, had my apartment broken into, and been investigated by the HRS. My friend committed suicide. I had the opportunity to ride in an ambulance not once but twice. I have heard the sound that a person's body makes when it is breaking, and had to cope with how it feels to be responsible but not at fault. In graduate school, both as a student and a parent, I learned that picking which battles to fight was vital to my survival in these roles, and that saying, "That was the hardest thing I have ever had to do," is just begging for something harder.

The majority of the first half of my life was spent in Lantana, Florida, where I won the Little Miss Lantana Contest when I was six. My father died in 1972. I attended Our

Savior Lutheran school where I skipped the fourth grade, Lantana Junior High School where I was voted most likely to succeed and Lake Worth High School where I was voted Girl of the Year of my Senior class. Nobody remembered that I skipped school to watch the filming of Body Heat. I applied to college at the University of Florida, much to my mother's chagrin. We argued, and I threatened to make fast food management my lifetime career if she did not let me go away to school. She relented, and we attended Preview together that summer.

I came to the University of Florida on August 15, 1982. Within a week, I met my first boyfriend and bounced my first check because of a bank error (they said I was $98,500 overdrawn). At one point, I held three jobs while I was in school just to make ends meet. I graduated in 1987, with a Bachelor's degree in Health Science and my first "real job" was at Shands Hospital as a Medical Technologist. In 1990, I landed a position with Baxter MicroScan, Inc. as a Technical Trainer. I moved to Sacramento, and this huge event in my life happened the day before they found the first body of what has come to be known as the Gainesville Murders. My mother was not happy that I had moved to California, but was thrilled I had moved from Gainesville. As a Technical Trainer, I learned that a corporate expense account is really quite a nuisance and that eating with obnoxious people is a waste of your time no matter how expensive the restaurant or who is paying the bill.

In 1992, I returned to Gainesville, this time in pursuit of graduate education. I received news that I was pregnant and accepted into graduate school the same week in June, 1993. My son Ryan was born on February 11, 1994, and in August of that year, I

began graduate school full time in the department of Pharmacodynamics. I am in awe of the education afforded me by both Ryan and Graduate School. And I am amazed to the degree that I have traveled full circle.

In 1982, I lived in campus housing in Rawlings Hall with my roommate Andrea. Now, Ryan and I live in family housing in Corry Village. In 1982, my idea of sewage was taking physics in the physics auditorium at 8:00 a.m. In 1998, the new physics building was built where the sewage treatment plant used to be. In 1982, it seemed like all of the computers for student use were on the fourth floor of Weil Hall. In 1998, every student was required to own one.

On a more personal front, in 1982, the last thing I wanted was to sound like my mother. In 1999, I marvel at the number of times I sound exactly like her. In 1982, my brother Scott was fighting for his life against cancer and was told he could never have children. In 1999, he and his wife have just adopted their second child. In 1982, my brother Jason began his school career, 1998 he began his retail career. In 1982, I wanted children and a career of my own. In 1999, my son will enter kindergarten and I begin my career as a scientist. I remember listening to the artist formerly known as Prince when he was Prince, singing "we're gonna party like its 1999". Well, 1999 is here, I am partying as Dr. Kelly Gridley, and I am ready for the next half of my life.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Maureen Keller-Wood

Maureen Keller-Wood, Chair
Associate Professor of
Pharmacodynamics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ralph Dawson, Jr.
Associate Professor of
Pharmacodynamics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Edwin Meyer
Associate Professor of
Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Professor of Pharmacodynamics

This dissertation was submitted to the Graduate Faculty of the Department of Pharmacodynamics of the College of Pharmacy and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1999

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